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(71) Applicant: **BAXTER AKTIENGESELLSCHAFT** [AT/AT]; Industriestrasse 67, A-1221 Vienna (AT).

(72) Inventors: **SCHEIFLINGER, Friedrich**; Michelbeuerngasse 4/17, A-1090 Vienna (AT). **KERSCHBAUMER, Randolf**; Peter Jordan Strasse 32-34/17, A-1190 Vienna (AT). **FALKNER, Falko-Guenter**; Neusiedlzeile 76A, A-2304 Orth/Donau (AT). **DORNER, Friedrich**; Peterlinigasse 17, A-1230 Vienna (AT).

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(54) Title: FACTOR IX/FACTOR IXa ANTIBODIES AND ANTIBODY DERIVATIVES

(57) Abstract: An antibody or antibody derivative against factor IX/activated factor IX (FIXa) which increases the procoagulant activity of FIXa.

The present invention relates to factor IX/factor IXa-antibodies and antibody derivatives.

Blood clots (thrombi) are formed by a series of zymogen activations referred to as the coagulation cascade. In the course of this enzymatic cascade, the activated form of each of such zymogens (referred to as factors) catalyzes the activation of the next one. Thrombi are deposits of blood components on the surface of a blood vessel wall and mainly consist of aggregated blood platelets and insoluble, cross-linked fibrin. Fibrin formation is effected by means of thrombin by limited proteolysis of fibrinogen. Thrombin is the final product of the coagulation cascade, (K.G. Mann, Blood, 1990, Vol. 76, pp.1-16).

Activation of factor X by the complex of activated factor IX (FIXa) and activated factor VIII (FVIIIa) is a key step in coagulation. The absence of the components of this complex or a disturbance of their function is associated with the blood coagulation disorder called hemophilia (J.E. Sadler & E.W. Davie: Hemophilia A, Hemophilia B and von Willebrand's disease, in G. Stamatoyannopoulos et al. (Eds.): The molecular basis of blood diseases. W.B. Saunders Co., Philadelphia, 1987, pp. 576-602). Hemophilia A denotes a (functional) absence of factor VIII activity, while Hemophilia B is characterized by the absence of factor IX activity. At present, treatment of Hemophilia A is effected via a substitution therapy by administering factor VIII concentrates. However, approximately 20-30% of Hemophilia A patients develop factor VIII inhibitors (i.e. antibodies against factor VIII), whereby the

effect of administered factor VIII preparations is inhibited. Treatment of factor VIII inhibitor patients is very difficult and involves risks, and so far there exist only a limited number of treatments for these patients.

In the case of patients having a low FVIII inhibitor level, it is possible, though expensive, to administer high doses of factor VIII to such patients and thus to neutralize the antibodies against factor VIII. The amount of factor VIII beyond that needed to neutralize the inhibitor antibodies then has hemostatic action. In many cases, desensitization can be effected, whereupon it is then possible again to apply standard factor VIII treatments. Such high dose factor VIII treatments require, however, large amounts of factor VIII, are time-consuming and may involve severe anaphylactic side reactions. Alternatively, the treatment may be carried out with porcine factor VIII molecules.

A further high-cost method involves removing factor VIII inhibitors through extra corporeal immunoadsorption on lectins which bind to immunoglobulins (protein A, protein G) or to immobilized factor VIII. Since the patient must be connected to an apheresis machine during this treatment, the treatment also constitutes a great burden on the patient. It is also not possible to treat an acute hemorrhage in this way.

At present, the therapy of choice is to administer activated prothrombin complex concentrates (APCC), such as FEIBA® and AUTOPLEX®, which are suitable for the treatment of acute hemorrhages even in patients having a high inhibitor titer (DE 31 27 318).

In the intravascular system of blood coagulation, the last step is the activation of factor X. This reaction is stimulated by the binding of factor VIIIa to factor IXa and the formation of a "tenase"-complex consisting of the factors IXa, VIIIa, X and phospholipid. Without the binding of FVIIIa, FIXa exhibits no or only a very slight enzymatic activity relative to FX.

Over the last several years, a number of possible binding sites for factor VIIIa to factor IXa have been characterized, and it has been shown that antibodies or peptides which bind to these regions inhibit the activity of FIXa (Fay et al., J. Biol. Chem., 1994, Vol.269, pp.20522-20527, Lenting et al., J. Biol. Chem., 1996, Vol. 271, pp. 1935-1940, Jorquera et al., Circulation, 1992, Vol. 86, Abstract 2725). The inhibition of coagulation factors, such as factor IX, has also been achieved through the use of monoclonal antibodies with the aim of preventing thrombosis formation (WO 97/26010).

The opposite effect, i.e. an increase in the factor IXa mediated activation of factor X, has been described by Liles D.K. et al., (Blood, 1997, Vol. 90, suppl. 1, 2054) through the binding of a factor VIII peptide (amino acids 698-712) to factor IX. Yet, this effect only occurs in the absence of factor VIIIa, while in the presence of factor VIIIa the factor IXa/factor VIIIa-mediated cleavage of factor X is inhibited by this peptide.

SUMMARY OF THE INVENTION

With a view to the possible risks and side effects which may occur in the treatment of hemophilia patients,

there is a need for a therapy which allows for the effective treatment of FVIII inhibitor patients. Therefore, it is an object of the present invention to provide a preparation for the treatment of blood coagulation disorders which has particular advantages for factor VIII inhibitor patients.

According to the present invention, this object is achieved through the use of antibodies or antibody derivatives against factor IX/factor IXa which have factor VIIIa-cofactor activity or factor IXa-activating activity and lead to an increase in the procoagulant activity of factor IXa. Surprisingly, the action of these inventive factor IX/factor IXa-activating antibodies and antibody derivatives is not negatively affected by the presence of inhibitors, such as inhibitors against factor VIII/factor VIIIa, but instead the procoagulant activity of factor IXa in this case also is increased.

A further advantage of this invention is that the administration of the preparation according to the invention allows for rapid blood coagulation even in the absence of factor VIII or factor VIIIa, even in the case of FVIII inhibitor patients. Surprisingly, these agents are also effective in the presence of factor VIIIa.

The antibodies and antibody derivatives according to the present invention thus have a FVIII-cofactor-like activity which, in a FVIII assay (e.g. a COATEST® assay or Immunochrom test) after 2 hours of incubation exhibits a ratio of background (basic noise) to measured value of at least 3. Calculation of this ratio may, e.g., be effected according to the following scheme:

Antibody measurement (OD 405) – blank value from reagent > 3
Mouse-IgG-measurement (OD 405) – blank value from reagent

after two hours of incubation.

The antibodies according to the invention preferably have an in vivo half life of at least 5 days, more preferably at least 10 days, though it is more preferred to have a half life of at least 20 days.

A further aspect of this invention is a preparation comprising antibodies and/or antibody derivatives against factor IX/factor IXa and a pharmaceutically acceptable carrier substance. Furthermore, the preparation according to the invention may additionally comprise factor IX and/or factor IXa.

A further aspect of the invention is the use of the antibodies or antibody derivatives to increase the amidolytic activity of factor Ixa.

Fig. 1 shows the results of a screening of supernatants from hybridoma cell cultures for FVIII-like activity. Pre-selected clones from fusion experiments, #193, #195 and #196, were tested in a chromogenic assay.

Fig. 2 shows the results of screening for IgG-mediated factor VIII-like activity in supernatants of a hybridoma cell culture of a master plate.

Fig. 3 shows the subcloning of clone 193/C0, namely the results of the first cloning round.

Fig. 4 shows a comparison of the chromogenic FVIII-like activity and factor IX-ELISA-reactivity of hybridoma cultures derived from the starting clone 193/C0.

Fig. 5 shows the results of the measurement of the chromogenic activity of some master clones and sub-clones.

Fig. 6A shows the FVIII-like activity of the anti-FIX/FIXa-antibodies 193/AD3 and 196/AF2 compared to human FVIII, TBS buffer and cell culture medium. After a lag phase, both antibodies gave rise to chromogenic substrate cleavage, as judged by the increasing optical density.

Fig. 6B shows a comparison of the chromogenic activity of factor VIII, 196/AF1, 198/AC1/1 and mouse-IgG.

Fig. 7A shows a comparison of the kinetics of Factor Xa generation by Factor VIII and 196/AF2 with and without the addition of a Factor Xa specific inhibitor.

Fig. 7B shows a comparison of the kinetics of the Factor Xa generation by Factor VIII, mouse-IgG and anti-factor IX/IXa-antibody 198/AM1 with and without the addition of a factor Xa-specific inhibitor, Pefabloc Xa®.

Fig. 8A shows a measurement of the dependence of the factor VIII-like activity of purified anti-factor IX/IXa-antibody 198/AC1/1 in the presence and absence of phospholipids, FIXa/FX and calcium ions.

Fig. 8B shows a measurement of the dependence of FXa generation by anti-FIXa-antibody 196/AF1 in the presence of phospholipids, Ca^{2+} in FIXa/FX.

Fig. 8C shows the generation of FXa by unspecific mouse IgG antibody.

Fig. 9 is a graphical representation of the coagulation times of Factor VIII-deficient plasma in an APTT assay by using various concentrations of anti-factor IX/IXa-antibody 193/AD3.

Fig. 10A shows that in the presence of Factor IXa, antibody 193/AD3 leads to a reduction in the coagulation time of factor VIII-deficient plasma.

Fig. 10B shows a dose-dependent reduction of the clotting time by antibody 193/AD3 in the presence of factor IXa- and factor VIII-inhibitors.

Fig. 11 shows the chromogenic activity of antibodies 198/A1, 198/B1 and 198/AP1 in the presence and absence of human FIXaβ.

Fig. 12 shows the primer sequences for the amplification of the genes of the variable heavy chain of mouse antibody.

Fig. 13 shows the primer sequences for the amplification of the genes of the variable light (kappa) chain of the mouse antibody.

Fig. 14 shows the DNA and derived protein sequence of the scFv from hybridoma cell line 193/AD3 (SEQ.ID.NOs. 81 and 82).

Fig. 15 shows the DNA and derived protein sequence of the scFv from hybridoma cell line 193/K2 (SEQ.ID.NOs. 83 and 84).

Fig. 16 shows the DNA and derived protein sequence of the scFv from hybridoma cell line 198/AB2 (subclone of 198/B1) (SEQ.ID.NOs. 85 and 86).

Fig. 17 shows the DNA and deduced protein sequence of scFv derived from the cell line 198/A1 (SEQ.ID.NOs. 87 and 88).

Fig. 18 demonstrates the chromogenic FVIII-like activity of peptide A1/3 in the presence of 2.9nM human FIXa. The scrambled version of peptide A1/3, peptide A1/5 does not give rise to any FXa generation.

Fig. 19 demonstrates the dependence of the chromogenic FVIII-like activity of peptide A1/3 on the presence of human FIXa. In the absence of human FIXa, peptide A1/3 does not give rise to any FXa generation. The buffer control, plain imidazole buffer is designated

IZ.

Fig. 20 shows that the chirality of Arg-residues does not play a significant role for the chromogenic activity of peptides A1/3-rd and A1/3-Rd-srmb.

Fig. 21 shows that the addition of 2.4µM peptide B1/7 to the reaction mixture led to a measureable generation of Fxa.

Fig. 22 shows that the addition of a FX-specific inhibitor results in a significant reduction in the reaction. If there was no FIXa and FX is added to the reaction mixture, no FXa was synthesized.

Fig. 23 shows vector pBax-IgG1.

Fig. 24 shows the increase of the amidolytic activity of FIXa in the presence of antibody 198/B1 (Fig. 24A) and IgM antibody 198/AF1 (Fig. 24B).

Fig. 25 demonstrates the chromogenic FVIII-like activity of the antibody 198/A1 Fab fragment in the presence of 2.3nM human FIXa. As a positive control the intact antibody 198/A1 was used as well as 7.5pM FVIII. The buffer control (IZ) was used as a negative control.

Fig. 26 shows the nucleotide and amino acid sequence of the 198AB2 scFv-alkaline phosphatase fusion protein (ORF of the expression vector pDAP2-198AB2#100, (SEQ.ID.NOs. 89 and 90).

The genes for the VL and the VH domains of antibody 198/AB2 (198/AB2 is an identical subclone of 198/B1) were derived from the corresponding hybridoma cells as described in example 10. The PCR product of the VH-gene was digested SfiI - AscI and the PCR-product of the VL-gene was digested AscI and NotI. VH and VL genes were linked via the AscI site and inserted into SfiI - NotI digested vector pDAP2 (Kerschbaumer R.J. et al, Immunotechnology 2, 145-150, 1996; GeneBank accession

No.:U35316). PelB leader: leader sequence of *Erwinia carotovora* Pectate Lyase B, His tag, Histidinee tag for metal ion chromatography.

Fig. 27 demonstrates the chromogenic FVIII-like activity of two antibody 198/B1 (subclone AB2) scFv fragment-alkaline phosphatase fusion proteins (198AB2#1 and 198AB2#100) in the presence of 2.3nM human FIXa. As a positive control 7.5pM FVIII was used.

Fig. 28 shows the amino acid and nucleotide sequence of pZip198AB2#102 (SEQ.ID.NOs. 91 and 92).

Fig. 29 shows the nucleotide and amino acid sequence of the mAB#8860 scFv-alkaline phosphatase fusion protein (vector pDAP2-8860scFv#11, (SEQ.ID.NOs. 93 and 94). The genes for the VL and the VH domains of antibody #8860 were derived from the corresponding hybridoma cells as described in example 10. The PCR product of the VH-gene was digested SfiI - AscI and the PCR-product of the VL-gene was digested AscI and NotI. VH and VL genes were linked via the AscI site and inserted into SfiI - NotI digested vector pDAP2 (Kerschbaumer R.J. et al, Immunotechnology 2, 145-150, 1996; GeneBank accession No.:U35316).

Fig. 30 shows the nucleotide and amino acid sequence of the mAB #8860 scFv-leucine zipper fusion protein (miniantibody; vector p8860-Zip#1.2, (SEQ.ID.NOs. 95 and 96). The gene of the scFv fragment was derived from mAB #8860 and was swapped from vector pDAP2-8860scFv#11 into SfiI-NotI digested plasmid pZip1 (Kerschbaumer R.J. et al., Analytical Biochemistry 249, 219-227, 1997; GeneBank accession No.: U94951)

Fig. 31 demonstrates the chromogenic FVIII-like activity of the 198/B1 (subclone AB2) miniantibody 198AB-Zip#102 in the presence of 2.3nM human FIXa. As a

positive control 4.8pM FVIII was used whereas a unrelated miniantibody (8860-Zip#1.2) and plain reaction buffer (IZ) served as negative controls.

Fig. 32 shows a schematic representation of the plasmid pMycHis6.

Fig. 33 shows the nucleotide and amino acid sequence of the part of the plasmid pMycHis6 differing from vector pCOCK (SEQ.ID.NOs. 97 and 98). Vector pMycHis6 was constructed by cleaving vector pCOCK (Engelhardt et al., 1994, Biotechniques, 17:44-46) with NotI and EcoRI and insertion of the oligonucleotides: mychis6-co: 5'ggccgcagaacaaaaactcatctcagaagaggatct gaatggggcggcacatcaccatcaccatcactaataag 3' (SEQ ID.No. 79) and mycchis-ic: 5'aattcttattagtgatggtgatggtgatgtgccgccccattcagatcctcttct gagatgagtttttgttctgc (SEQ.ID.No. 80).

Fig. 34 shows the nucleotide and amino acid sequence of 198AB2 scFv (linked to the c-myc-tag and the His6- tag): ORF of the expression vector pMycHis6-198AB2#102. Vector pMycHis6 was constructed by cleaving vector pCOCK (Engelhardt O. et al, BioTechniques 17, 44-46, 1994) NotI - EcoRI and inserting the following annealed oligonucleotides:
(5'-GGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCTGAATGGG GCGGCACATCACCATCACCATCACTAATAAG - 3' (SEQ.ID.No. 103) and
5'- TTATTAGTGATGGTGATGGT GATGTGCCGCCCCATTCAGATCCTCTTCTGAGATGAGTTTTTGTTCTGC-3'(SEQ.ID.NO. 104)). The resultant vector, named pMycHis6, was cleaved SfiI - NotI and the gene of scFv 198AB2 was swapped into this vector from vector pDAP2-198AB2#100.

Fig. 35 shows the nucleotide and amino acid

sequence of the mAB #8860 scFv linked to the c-myc-tag and the His6- tag (vector p8860-M/H#4c, SEQ.ID.NOs. 101 and 102). Plasmid pMycHis6 was cleaved with SfiI and NotI and the DNA sequence coding for the scFv 8860#11 protein was inserted from pDAP2-8860scFv#11 (see Fig.29) yielding plasmid p8860-M/H#4c.

Fig. 36 demonstrates the chromogenic FVIII-like activity of the 198/B1 (subclone AB2) scFv fragment (MycHis-198AB2#102) in the presence of 2.3nM human FIXa. As a positive control 4.8pM FVIII was used whereas a unrelated scFv (8860-M/H#4c) and plain reaction buffer (IZ) served as negative controls.

Antibodies and Antibody Derivatives.

The present invention also comprises the nucleic acids encoding the inventive antibodies and antibody derivatives, expression vectors, hybridoma cell lines, and methods for producing the same.

Antibodies are immunoglobulin molecules having a specific amino acid sequence which only bind to antigens that induce their synthesis (or its immunogen, respectively) or to antigens (or immunogens) which are very similar to the former. Each immunoglobulin molecule consists of two types of polypeptide chains. Each molecule consists of large, identical heavy chains (H chains) and two light, also identical chains (L chains). The polypeptides are connected by disulfide bridges and non-covalent bonds. In vivo, the heavy and light chains are formed on different ribosomes, assembled in the cell, and secreted as intact immunoglobulins (Roitt I. et al., in: Immunology, second ed., 1989).

The inventive antibodies and antibody derivatives and organic compounds derived there from comprise human

and animal monoclonal antibodies or fragments thereof, single chain antibodies and fragments thereof and miniantibodies, bispecific antibodies, diabodies, triabodies, or di-, oligo- or multimers thereof. Also included are peptidomimetics or peptides derived from the antibodies according to the invention, e.g. they comprise one or several CDR regions, preferably the CDR3 region.

Further included are human monoclonal antibodies and peptide sequences which, based on a structure activity connection, are produced through an artificial modeling process (Greer J. et al., J. Med. Chem., 1994, Vol. 37, pp. 1035-1054).

The term factor IX/IXa activating antibodies and antibody derivatives may also include proteins produced by expression of an altered, immunoglobulin-encoding region in a host cell, e.g. "technically modified antibodies" such as synthetic antibodies, chimeric or humanized antibodies, or mixtures thereof, or antibody fragments which partially or completely lack the constant region, e.g. Fv, Fab, Fab' or $F(ab)'_2$ etc. In these technically modified antibodies, e.g., a part or parts of the light and/or heavy chain may be substituted. Such molecules may, e.g., comprise antibodies consisting of a humanized heavy chain and an unmodified light chain (or chimeric light chain), or vice versa. The terms Fv, Fc, Fd, Fab, Fab' or $F(ab)_2$ are used as described in the prior art (Harlow E. and Lane D., in "Antibodies, A Laboratory Manual", Cold Spring Harbor Laboratory, 1988).

The present invention also comprises the use of Fab fragments or $F(ab)_2$ fragments which are derived from monoclonal antibodies (mAb), which are directed against

factor IX/factor IXa and cause an increase of the procoagulant activity of factor IXa.

Preferably, the heterologous framework regions and constant regions are selected from the human immunoglobulin classes and isotypes, such as IgG (subtypes 1 to 4), IgM, IgA and IgE. In the course of the immune response, a class switch of the immunoglobulins may occur, e.g. a switch from IgM to IgG; therein, the constant regions are exchanged, e.g. from μ to γ. A class switch may also be caused in a directed manner by means of genetic engineering methods ("directed class switch recombination"), as is known from the prior art (Esser C. and Radbruch A., Annu. Rev. Immunol., 1990, Vol. 8, pp. 717-735). However, the antibodies and antibody derivatives according to the present invention need not comprise exclusively human sequences of the immunoglobulin proteins.

In one particular embodiment, a humanized antibody comprises complement determining regions (CDRs) from murine monoclonal antibodies which are inserted in the framework regions of selected human antibody sequences. However, human CDR regions can also be used. Preferably, the variable regions in the human light and heavy chains are technically altered by one or more CDR exchanges. It is also possible to use all six CDRs or varying combinations of less than six CDRs.

The humanized antibody according to the present invention preferably has the structure of a human antibody or of a fragment thereof and comprises the combination of characteristics necessary for a therapeutic application, e.g., the treatment of coagulation disorders in patients, preferably factor VIII inhibitor patients.

A chimeric antibody differs from a humanized antibody in that it comprises the entire variable regions including the framework regions of the heavy and light chains of non-human origin in combination with the constant regions of both chains from human immunoglobulin. A chimeric antibody consisting of murine and human sequences may, for example, be produced.

According to the present invention, the antibodies and antibody derivatives may also be single chain antibodies or miniantibodies (scFv fragments, which, e.g., are linked to proline-rich sequences and oligomerisation domains, e.g. Pluckthun A. and Pack P., Immunotechnology, 1997, Vol. 3, pp. 83-105) or single chain Fv (sFv) which incorporate the entire antibody binding region in one single polypeptide chain. For instance, single chain antibodies may be formed by linking the V-genes to an oligonucleotide which has been constructed as a linker sequence and connects the C terminus of the first V region with the N terminus of the second V region, e.g. in the arrangement VH-Linker-VL or VL-Linker-V_H; both, V_H and V_L thus may represent the N-terminal domain (Huston JS et al., Int. Rev. Immunol., 1993, Vol. 10, pp. 195-217; Raag R. and Whitlow M., FASEB J., 1995, Vol. 9, pp. 73-80). The protein which can be used as linker sequence may, e.g., have a length of up to 150 Å, preferably up to 80 Å, and more preferably up to 40 Å. Linker sequences containing glycine and serine are particularly preferred for their flexibility, or glutamine and lysine, respectively, for their solubility. The choice of the amino acid is effected according to the criteria of immunogenicity and stability, also depending on whether or not these single chain antibodies are to be suitable for physiological or

industrial applications (e.g. immunoaffinity chromatography). The single chain antibodies may also be present as aggregates, e.g. as trimers, oligomers or multimers. The linker sequence may, however, also be missing, and the connection of the V_H and V_L chains may occur directly.

Bispecific antibodies are macromolecular, heterobifunctional cross-linkers having two different binding specificities within one single molecule. In this group belong, e.g., bispecific (bs) IgGs, bs IgM-IgAs, bs IgA-dimers, bs $(Fab')_2$, bs$(scFv)_2$, diabodies, and bs bis Fab Fc (Cao Y. and Suresh M.R., Bioconjugate Chem., 1998, Vol. 9, pp. 635-644).

By peptidomimetics, protein components of low molecular weight are understood which imitate the structure of a natural peptide component, or of templates which induce a specific structure formation in an adjacent peptide sequence (Kemp DS, Trends Biotechnol., 1990, pp. 249-255). The peptidomimetics may, e.g., be derived from the CDR3 domains. Methodical mutational analysis of a given peptide sequence, i.e. by alanine or glutamic acid scanning mutational analysis, allows for the identification of peptide residues critical for procoagulant activity. Another possibility to improve the activity of a certain peptide sequence is the use of peptide libraries combined with high throughput screening.

The term antibodies and antibody derivatives may also comprise agents which have been obtained by analysis of data relating to structure-activity relationships. These compounds may also be used as peptidomimetics (Grassy G. et al., Nature Biotechnol.,

1998, Vol. 16, pp. 748-752; Greer J. et al., J. Med. Chem., 1994, Vol. 37, pp. 1035-1054).

Examples of hybridoma cells expressing the antibodies or antibody derivatives according to the invention were deposited on 9 September 1999 under the numbers 99090924 (#198/A1), 99090925 (#198/B1) and 99090926 (#198/BB1) and on December 16, 1999 under the numbers 99121614 (#193/A0), 99121615 (#196/C4), 99121616 (#198/D1), 99121617 (198/T2), 99121618 (#198/G2), 99121619 (#198/AC1) and 99121620 (#198/U2) according to the Budapest Treaty.

Methods of Production:

The antibodies of the present invention can be prepared by methods known from the prior art, e.g. by conventional hybridoma techniques, or by means of phage display gene libraries, immunoglobulin chain shuffling or humanizing techniques (Harlow E. and Lane D., in: Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, 1988). The production of the inventive antibodies and antibody derivatives may , for instance, be made by conventional hybridoma techniques (Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, 1988, Eds. Harlow and Lane, pp. 148-242). According to the present invention, human and also non-human species may be employed therefor, such as cattle, pigs, monkeys, chickens and rodents (mice, rats). Normal, immunocompetent Balb/c mice or FIX-deficient mice may, e.g., be used (factor IX-deficient mice may be obtained from Dr. Darrel Stafford from the University of North Carolina, Chapel Hill). Immunization may, e.g., be effected with factor IX, factor IXaα or completely activated factor IXaβ, or with fragments thereof.

The hybridomas are selected with a view to the fact that the antibodies and antibody derivatives in the supernatants of the hybridoma cells bind to factor IX/factor IXa and cause an increase of the procoagulant activity of factor IXa. The increase in the procoagulant activity may, e.g., be proven by assaying methods as known from the prior art for the measurement of factor VIII-like activity, e.g. chromogenic assays.

Alternatively, the antibodies and antibody derivatives of the invention may also be produced by recombinant production methods. In doing so, the DNA sequence of the antibodies according to the invention can be determined by known techniques, and the entire antibody DNA or parts thereof can be expressed in suitable systems. Recombinant production methods can be used, such as those involving phage display, synthetic and natural libraries, expression of the antibody proteins in known expression systems, or expression in transgenic animals (Jones et al., Nature, 1986, Vol. 321, pp.522-525; Phage Display of Peptides and Proteins, A Laboratory Manual, 1996, Eds. Kay et al., pp. 127-139; US 4,873,316; Vaughan T.J. et al., Nature Biotechnology, 1998, pp. 535-539; Persic L. et al., Gene, 1997, pp. 9-18; Ames R.S. et al., J.Immunol.Methods, 1995, pp. 177-186).

The expression of recombinantly produced antibodies may be effected by means of conventional expression vectors, such as bacterial vectors, such as pBr322 and its derivatives, pSKF or eukaryotic vectors, such as pMSG and SV40 vectors. Those sequences which encode the antibody may be provided with regulatory sequences which regulate the replication, expression and secretion from

the host cell. These regulatory sequences comprise promoters, e.g. CMV or SV40, and signal sequences.

The expression vectors may also comprise selection and amplification markers, such as the dihydrofolate reductase gene (DHFR), hygromycin-B- phosphotransferase, thymidine-kinase etc.

The components of the vectors used, such as selection markers, replicons, enhancers etc., may either be commercially obtained or prepared by means of conventional methods. The vectors may be constructed for the expression in various cell cultures, e.g. for mammalian cells such as CHO, COS, fibroblasts, insect cells, yeast or bacteria, such as E. coli. Preferably, those cells are used which allow for an optimal glycosylation of the expressed protein. Particularly preferred is the vector pBax (cf. Fig. 17) which is expressed in CHO cells or in SK-Hep.

The production of Fab fragments or $F(ab)_2$ fragments may be effected according to methods known from the prior art, e.g. by cleaving a mAb with proteolytic enzymes, such as papain and/or pepsin, or by recombinant methods. These Fab and $F(ab)_2$ fragments may also be prepared by means of a phage display gene library (Winter et al., 1994, Ann. Rev. Immunol., 12:433-455).

The antibody derivatives may also be prepared by means of methods known from the prior art, e.g. by molecular modeling, e.g. from Grassy G. et al., Nature Biotechnol., 1998, Vol. 16, pp. 748-752, or Greer J. et al., J. Med. Chem., Vol. 37, pp. 1035-1054, or Rees A. et al., in: "Protein Structure Prediction: A practical approach", ed. Sternberg M.J.E., IRL press, 1996, chapt. 7-10, pp. 141-261.

The purification of the inventive antibodies and antibody derivatives may also be carried out by methods described in the prior art, e.g., by ammonium sulfate precipitation, affinity purification (protein G-Sepharose), ion exchange chromatography, or gel chromatography. The following methods may be used as the test methods to show that the antibodies and antibody derivatives of the present invention bind to factor IX/factor IXa, increase the procoagulant activity of factor IXa or have factor VIII-like activity.: the one step coagulation test (Mikaelsson and Oswaldson, Scand. J. Haematol., Suppl., 33, pp. 79-86, 1984) or the chromogenic tests, such as COATEST VIII:C® (Chromogenix) or Immunochrom (IMMUNO). In principle, all the methods used for determining factor VIII activity may be used. As the control blank value for the measurements, e.g., unspecific mouse-IgG antibody may be used.

The present antibodies and antibody derivatives are suitable for therapeutic use in the treatment of coagulation disorders, e.g. in the case of hemophilia A, for factor VIII inhibitor patients etc. Administration may be effected by any method suitable to effectively administer the therapeutic agent to the patient, e.g. by oral, subcutaneous, intramuscular, intravenous or intranasal administration.

Therapeutic agents according to the invention may be produced as preparations which comprise a sufficient amount of antibodies or of antibody derivatives as the active agent in a pharmaceutically acceptable carrier substance. These agents may be present either in liquid or in powderized form. Moreover, the preparations according to the invention may also comprise mixtures of different antibodies, the derivatives thereof and/or

organic compounds derived therefrom, as well as mixtures consisting of antibodies and factor IX and/or factor IXa. Factor IXa may be present as factor IXaα and/or factor IXaβ. An example of an aqueous carrier substance is, e.g., saline. The solutions are sterile, sterilisation being effected by conventional methods.

The antibodies or antibody derivatives according to the invention may be present in lyophilized form for storage and be suspended in a suitable solvent before administration. This method has proven generally advantageous for conventional immunoglobulins, and known lyophilisation and reconstitution methods may be applied in this case.

Moreover, the antibodies and antibody derivatives according to the invention may also be used for industrial applications, e.g. for the purification of factor IX/factor IXa by means of affinity chromatography, or as a component of detection methods (e.g. ELISA assays), or as an agent for identification of and interaction with functional domains of a target protein.

The present invention will be described in more detail by way of the following examples and drawing figures, to which, however, it shall not be restricted.

Examples

Example 1: Immunization of immunocompetent mice and generation of anti-FIX/IXa antibody secreting hybridoma cells

Groups of 1-3 normal immunocompetent 5-8 week old Balb/c mice were immunized with 100µg antigen (100µl doses) via the intraperitoneal (i.p.) route. In a typical experiment, mice were inoculated with either

recombinant human coagulation factor (F) IX (Benefix^{TM}), human activated FIXaα (Enzyme Research Laboratories, Lot: FIXaα 1190L) or human FIXaβ (Enzyme Research Laboratories, Lot: HFIXAaβ 1332 AL,) adjuvanted with $Al(OH)_3$ or KFA.

Individual mice were boosted at various times with 100µg antigen (100µl doses, i.p) and sacrificed two days later. Spleen cells were removed and fused to P3 X63-Ag8 6.5.3 myeloma cells essentially as described by Lane et al., 1985 (J. Immunol. Methods, Vol. 81, pp. 223-228). Each fusion experiment was individually numbered, i.e. #193, 195, 196 or 198.

Hybridoma cells were grown in 96 well plates on a macrophage feeder layer (app. 10^5 cells/ml) and selected in HAT-medium (RPMI-1640 medium supplemented with antibiotics, 10% FCS, Na-pyruvate, L-glutamine, 2-mercaptoethanol and HAT (HAT 100x: $1.0x10^{-2}M$ hypoxanthine in H_2O (136.1 mg/100ml H_2O), $4.0x10^{-5}M$ aminopterin in H_2O (1.76 mg/100ml H_2O) and $1.6x10^{-3}M$ thymidine in H_2O (38.7 mg/100ml H_2O). Medium was first changed after 6 days and thereafter twice a week. After 2-3 weeks HAT-medium was changed to HT-medium (RpMI-1640 supplemented with antibiotics, 10%FCS, Na-pyruvate, L-glutamine, 2-mercaptoethanol and HT) and later on (after additional 1-2 weeks) to normal growth medium (RPMI-1640 medium supplemented with 10%FCS, Na-pyruvate, L-glutamine and 2-mercaptoethanol) (see: HYBRIDOMA TECHNIQUES, EMBO, SKMB Course 1980, Basel).

In another set of experiments FIX deficient C57B16 mice (Lin et al., 1997, Blood, 90:3962) were used for immunization and subsequent hybridoma production. Since FIX knockout (k.o.) mice do not express endogenous FIX,

the anti (a)-FIX antibody spectrum achievable is supposed to be different compared to normal Balb/c mice (due to lack of tolerance)

Example 2: Assaying for FVIII-like activity in supernatants of anti-FIX/FIXa antibody secreting hybridoma cells

In order to assay the FVIII-like activity of anti-FIXa antibodies secreted by hybridoma cells, the commercially available test-kit COATEST VIII:C/4® (Chromogenix) was employed. The assay was done essentially as described by the manufacturer with the following modifications:

To allow high throughput screening, the assay was downscaled to microtiter plate format. Briefly, 25µl aliquots of hybridoma supernatants were transferred to microtiter plate (Costar, #3598) wells and warmed to 37°C. Chromogenic substrate (S-2222), synthetic thrombin inhibitor (I-2581), factor (F) IXa and FX were reconstituted in sterile water and FIXa/FX was mixed with phospholipids according to the supplier's protocol. Per reaction, 50µl of the phospholipid/FIXa/FX solution were combined with 25µl $CaCl_2$ (25mM) and 50µl of the substrate/inhibitor cocktail. To start the reaction, 125µl of the premix were added to the hybridoma supernatant in the microtiter plates and incubated at 37°C. Absorbency at 405nm and 490nm of the samples was read at various times (30min to 12h) against a reagent blank (MLW, cell culture medium instead of hybridoma supernatant) in a Labsystems iEMS Reader MF^{TM} microtiter plate reader. FVIII-like activity of the samples was calculated by comparing the absorbency of the samples against the absorbency of a diluted FVIII reference standard (IMMUNO AG # 5T4AR00) using GENESIS^{TM} software.

The results of a screening for FVIII-like activity in hybridoma cell culture supernatants are shown in Fig.

1. Pre-selected clones derived from fusion experiments #193, #195 and #196 (see above) were examined in a chromogenic FVIII assay as described. Clones 193/M1, 193/N1 and 193/P1 are subclones derived from the master clone 193/C0 (see below). Master clone 195/10 was derived from fusion experiment #195 and clones 196/A0, 196/B0 and 196/C0 were derived from fusion experiment #196. In a typical screening experiment, approximately 1000 clones (in 96 wells) from a single fusion experiment were pre-screened for FVIII-like activity. Subsequently, selected clones were grown on a larger scale (3-5 ml supernatant) and re-analyzed in a chromogenic assay. As a negative control cell culture medium was assayed on each plate (MLW).

Wells either exhibiting high FVIII-like activity or substantial FVIII-like activity were subjected to subcloning procedures. The selection and subcloning process is exemplified for the screening and subcloning of an IgG producing cell line (i.e. 193/C0) but has been done exactly the same way for an IgM (i.e. 196/C0, see below, Fig. 5) producing clone.

The selection process was done by initially plating all hybridoma cell clones derived from a single fusion experiment on ten 96 well plates thereby creating the so called "master plates". Singular positions (wells) on a master plate usually contained more than one hybridoma cell clone (usually 3 to 15 different clones). Subsequently, the antibody secreted by only several thousand cells was tested. These cells grew under conditions suboptimal for antibody production, which is known to be best in dying cells. So the expected specific anti-FIX antibody concentration in the supernatant may be in the range of 10^{-12} to 10^{-14} M.

This explains why incubation periods had to be extended compared to standard FVIII assays.

Results of a screening for an IgG mediated FVIII-like activity in hybridoma cell culture supernatants of a master plate are shown in Fig. 2. Supernatants were examined in a chromogenic FVIII assay. Shown are the results derived from the fifth master plate of fusion experiment number #193 (Balb/c mice immunized with FIXaα). Absorbance was read after 4 hours of incubation at 37°C. Position ES was identified as exhibiting FVIII like activity significantly higher than the blank (MLW). This cell pool was designated 193/C0 and was further subcloned (Figure 3). As each well of the master plate contains more than one hybridoma cell clone, cells of a single positive well were expanded and plated at a calculated cell density of 2 - 0.2 cells/well on a 96 well plate. Again, the supernatants were tested for FVIII-like activity and positive positions were subjected to another round of subcloning. Typically three to four rounds of subcloning were performed with each clone displaying FVIII-like activity to obtain homogenous cell populations. Here the results of the chromogenic assay of the 193/C0 subclones are shown. Absorbance was read after a 4 hour incubation period at 37°C. Positions A6 and D5 exhibited substantial FVIII-like activity and were named 193/M1 and 193/P1, respectively. These two clones were subjected to another round of subcloning. As a negative control plain cell culture medium was assayed on each plate (MLW(H1)).

A comparison of chromogenic FVIII-like activity and FIX-ELISA reactivity of small scale (3 ml) hybridoma cultures is shown in Fig. 4. Before a decision was made whether a master clone (or subclone) was to be further

subcloned, clones were grown at a 3-5 ml scale and the supernatants were checked again. This graph shows the FIX specific ELISA results and the FVIII-like chromogenic activity of the master clone 193/C0 and all its subclones which were identified as positives and re-checked. Blanks (absorbency of the chromogenic reagent itself) were subtracted in the case of the ELISA as well as the chromogenic assay readings depicted here. Clone 193/M1 was subcloned and yielded clones 193/V2, 193/M2 and 193/U2. The other clones of the 2nd round came from 193/P1, 193/AB2 and 193/P2 were subcloned. 193/AF3, 193/AB3 and 193/AE3 are subclones of 193/AB2. The other clones of the 3rd round came from 193/P2. Finally 193/AF3 (→193/AF4), AE3 (→193/AE4, 193/AL4, 193/AN4 and 193/AO4) and 193/AD3 (→193/AG4, 193/AH4, 193/AD4, 193/AI4, 193/AK4) were subcloned.

From each fusion experiment, several (5-15) master clones (selected from the master plate) were identified and subjected to subcloning. After 3 rounds of sub-cloning, most of the cell lines were homogenous as demonstrated by ELISA and chromogenic activity analysis (see Fig. 4) as well as by cDNA sequence analysis. A specific master clone and all its subclones produce the same FIX/FIXa binding antibody. However, there are huge differences in the antibody protein sequences of clones derived from different master clones (see Example 11). Most hybridoma cell lines express antibodies from the IgG subclass (i.e. clones #193, #198, like 198/A1, 198/B1, 198/BB1). However, we were also able to select some clones expressing IgM antibodies.

The chromogenic activity of hybridoma supernatant of some important master clones and subclones was determined. Absorbance was measured after a 1h 30 min

and 3h 30 min incubation period at 37°C (Fig. 5). In contrast to all the clones from the 193rd fusion, clone 196/C0 and its subclone 196/AP2 produced a FIX/FIXa-specific IgM antibody that gave a strong chromogenic activity even after a short period of incubation.

The following cell lines have been deposited with the European Collection of Cell Cultures (ECACC) in accordance with the Budapest Treaty: 98/B1 (ECACC No. 99090925); 198/A1 (ECACC No. 99090924); 198/BB1 (ECACC No. 99090926); 193/AO (ECACC No. 99121614); 196/C4 (ECACC No. 99121615); 198/D1 (ECACC No. 99121616); 198/T2 (ECACC No. 99121617); 198/G2 (ECACC No. 99121618); 198/AC1 (ECACC No. 99121619); and 198/U2 (ECACC No. 99121620).

To do a more in depth analysis of the biochemical properties of certain antibodies, homogenous hybridoma cell lines expressing different antibodies with FVIII-like activity were expanded and used to express the antibody in question on a larger scale (100-1000 ml). These antibodies were affinity purified (see Example 3) prior to being used in further experiments.

Example 3: Factor IX/FIXa$_{(\alpha,\beta)}$ binding properties of antibodies exhibiting FIX/FIXa activating activity

Factor IX and the two activated forms of FIX, FIXaα and FIXaβ (FIX/FIXa$_{(\alpha,\beta)}$) were diluted in TBS (25mM Tris HCl, 150mM NaCl, pH 7.5) to a final concentration of 2µg/ml. Nunc Maxisorp ELISA plates were coated with 100µl FIX/FIXa$_{(\alpha,\beta)}$ solution according to standard procedures (4°C, overnight) and washed several times with TBST (TBS, 0.1% (v/v) Tween 20). 50µl hybridoma supernatant was diluted 1:1 with 50µl TBST/2%BSA and added to the coated ELISA plate. After an incubation

period of 2h at room temperature (RT), plates were washed 4 times with TBST and incubated (2h, RT) with 100µl/well of a 1:25000 dilution (in TBST/1%BSA) of an anti-mouse IgG (Fc-specific) peroxidase conjugated antibody (Sigma, #A-0168). Wells were washed 5 times with TBST and finally stained with 100µl freshly prepared staining solution (10ml 50mM sodium citrate, pH 5 supplemented with 100µl OPD (60mg OPD/ml) and 10µl 30% H_2O_2). The reaction was stopped by the addition of 50ml H_2SO_4 and the optical density recorded at 492nm and 620nm in a Labsystems iEMS Reader MF^{TM} microtiter plate reader employing GENESIS^{TM} software.

In certain cases, instead of an anti-mouse IgG ELISA, an anti- mouse IgM ELISA was carried out.

Purification of mouse-IgG from hybridoma cell culture supernatants

Hybridoma supernatant (100-500 ml) was supplemented with 200 mM Tris/HCl buffer (pH 7.0) and solid NaCl to give final concentrations of 20 mM Tris and 3M NaCl, respectively. The supernatant was then clarified by centrifugation at 5500 x g for 10 minutes. A 1 ml protein G affinity chromatography column (Protein G Sepharose Fast Flow, Amersham-Pharmacia) was washed with 15 ml 20 mM Tris/Cl pH 7.0 and afterwards equilibrated with 10 ml of 20 mM Tris/Cl buffer pH 7.0 containing 3M NaCl. The hybridoma supernatant containing 3M NaCl was then loaded onto the column by gravity. The column was washed with 15 ml of 20 mM Tris/Cl buffer, pH 7.0, containing 3M NaCl. Bound IgG was further eluted with 12 ml glycine/HCl buffer pH 2.8 and 1 ml fractions were collected. 100µl of 1M Tris pH 9.0 were added to each fraction for neutralization. Fractions containing the

IgG were identified by mixing 50µl with 150µl of a staining solution (BioRad concentrate, 1:5 diluted with water) in wells of a microplate. Positive fractions were pooled, concentrated to 1 ml in an ultrafiltration concentrator device (Centricon Plus 20, Amicon) according to the manufacturer. The concentrate was diluted with 19 ml TBS (20 mM Tris/Cl buffer pH 7.0 containing 150mM NaCl) and again concentrated to 1 ml. The diluting-concentrating step was repeated for two more times in order to bring IgG into TBS.

Purification of mouse-IgM from hybridoma cell supernatants

100-500 ml of hybridoma cell culture supernatant were concentrated to 5-10 ml either with an ultra-filtration concentrator device (Centricon Plus 20, Amicon) according to the manufacturer or by ammonium sulfate precipitation (40% saturation, 0°C) and redissolving the precipitate with 5-10 ml of TBS. In either case the concentrate was dialyzed against 20mM Tris Cl buffer pH 7.4 containing 1.25M NaCl and further concentrated to 1 ml in a Centricon Plus 20, (Amicon) ultrafiltration device. IgM was purified from this concentrate with the ImmunoPure IgM Purification Kit (Pierce) according to the manufacturer. Fractions collected during elution from the maltose binding protein-column were tested for IgM, pooled, concentrated and brought into TBS as described for IgG.

Determination of IgG concentrations in purified preparations

Total IgG content 280nm - extinction of appropriate dilutions were measured. E280 = 1.4 corresponds to 1 mg/ml protein.

Factor IXa specific IgG (quantitative ELISA)

Wells of a microplate (Nunc Maxisorp) were incubated with 2µg/ml factor IXa diluted in TBS (25mM Tris/HCl pH 7.5 containing 150mM NaCl) overnight at 4°C. Wells were washed four times with TBST (25mM Tris/HCl pH 7.5 containing 150mM NaCl and 0.1% (v/v) Tween 20). As a standard monoclonal AB the HIX1 anti-FIX (accurate) was used. Standard and samples were diluted in TBST containing 2%(w/v) BSA. The standard dilution series and appropriate dilutions of the samples were incubated on the ELISA-plate for 2 hours at room temperature. Plates were washed 4 times with TBST and incubated (2h, RT) with 100µl/well of a 1:25000 dilution (in TBST/1%BSA) of an anti-mouse IgG (Fc-specific) peroxidase conjugated antibody (Sigma, #A-0168) FIXa. Wells were washed 5 times with TBST and finally stained with 100µl freshly prepared staining solution (10ml 50mM sodium citrate, pH 5 supplemented with 100µl OPD (60mg OPD/ml) and 10µl 30% H_2O_2). The reaction was stopped by the addition of 50ml H_2SO_4 and after 30 minutes the optical density was recorded at 492nm and 620nm in a Labsystems iEMS Reader MF^{TM} microtiter plate reader employing GENESIS^{TM} software.

Example 4: Anti-FIX/FIXa antibodies exhibiting FVIII-like activity in a chromogenic FVIII assay

Several anti-FIX/FIXa antibody producing hybridoma clones were subcloned up to four times and the resulting monoclonal hybridoma cell line used to produce monoclonal antibody containing supernatant. IgG isotype antibodies derived from these supernatants were purified over affinity columns and dialyzed against TBS (see above). IgM antibodies were used as unpurified supernatant fractions. The following experiments were

done with two sets of representative antibodies: 193/AD3 and 198/AC1/1 (IgG isotype, the antibody 198/AC1/1 is a preparation from the parent 198/AC1 hybridoma clone, i.e. that a (frozen) vial containing 198/AC1 cells is cultivated and antibodies are produced. The supernatant is then used for these experiments.) and 196/AF2 and 196/AF1 (IgM isotype) (Fig. 6A and Fig. 6B). Briefly, 25µl aliquots of monoclonal antibody containing sample (unpurified hybridoma supernatant or, where indicated, a certain amount of FIX specific antibody) were transferred to microtiter plate wells and warmed to 37°C. Chromogenic substrate (S-2222), synthetic thrombin inhibitor (I-2581), factor (F) IXa and FX were reconstituted in sterile water and FIXa/FX was mixed with phospholipids according to the supplier's protocol. Per reaction, 50µl of the phospholipid/FIXa/FX solution were combined with 25µl $CaCl_2$ (25mM) and 50µl of the substrate/inhibitor cocktail. To start the reaction, 125µl of the premix were added to the monoclonal antibody solution in the microtiter plates and incubated at 37°C. Absorbance at 405nm and 490nm of the samples was read at various times (5min to 6h) against a reagent blank (cell culture medium instead of hybridoma supernatant) in a Labsystems iEMS Reader MF^{TM} microtiter plate reader using GENESIS^{TM} software.

The time course of FVIII-like activity exhibited by monoclonal antibodies 193/AD3 (IgG isotype) and 196/AF2 (IgM isotype) compared to human FVIII (12 and 16mU/ml), TBS and to cell culture medium is shown in Fig. 6A. After a lag phase, both antibodies give rise to chromogenic substrate cleavage, as judged by the

increasing optical density measurable at 405nm wavelength.

The time course of FVIII-like activity exhibited by monoclonal antibodies 198/AC1/1 (IgG isotype, 10µg/ml) and 196/AF1 (IgM isotype, unpurified supernatant) compared to human FVIII (16mU/ml) and 10µg/ml of mouse IgG is shown in Fig. 6B. After a lag phase, both antibodies give rise to chromogenic substrate cleavage, as judged by the increasing optical density measurable at 405nm wavelength.

Example 5: FVIII-like activity exhibited by anti-FIX/FIXa-antibodies generates factor Xa and is phospholipid, FIXa/FX and Ca^{2+} dependent.

Factor VIII activity is usually determined with a chromogenic assay and/or an APTT-based clotting assay. Both types of assays rely on FVIIIa/FIXa-mediated factor Xa generation. In the case of a chromogenic FVIII assay, the factor Xa produced will subsequently react with a chromogenic substrate, which can be monitored spectroscopically, e.g., in an ELISA reader. In an APTT based clotting assay free factor Xa will assemble with FVa on a phospholipid surface in the so-called prothrombinase complex and activate prothrombin to thrombin. Thrombin in turn gives rise to fibrin generation and finally to clot formation. Central to the two assay systems is generation of factor Xa by the FVIIIa/FIXa complex.

To demonstrate that the FVIII-like activity exhibited by anti-FIX/FIXa-antibodies indeed generates factor Xa, the following experiment was carried out. Several 25µl aliquots of unpurified hybridoma supernatant 196/AF2 (IgM isotype) were transferred to microtiter plate wells and warmed to 37°C. As a positive control, 16mU of

Recombinate™ were diluted into hybridoma medium (196 HM 007/99) and treated exactly the same way as the hybridoma supernatant. As a negative control, plain hybridoma medium was used. Chromogenic substrate (S-2222), synthetic thrombin inhibitor (I-2581), factor IXa and FX were reconstituted in sterile water and FIXa/FX was mixed with phospholipids according to the supplier's protocol. Pefabloc Xa®, a factor Xa specific proteinase inhibitor (Pentapharm, LTD), was reconstituted with water to a final concentration of 1mM/l. Per reaction, 50µl of the phospholipid/FIXa/FX solution were combined with 25µl $CaCl_2$ (25mM) and 50µl of the substrate/ thrombin-inhibitor cocktail. To start the reaction, 125µl of the premix were added to the samples in the microtiter plates and incubated at 37°C. Where indicated, 35µM Pefabloc Xa® were added. Absorbance at 405nm and 490nm was read at various times (every 5 minutes to 6h) against a reagent blank (cell culture medium) in a Labsystems iEMS Reader MF™ microtiter plate reader employing the GENESIS™ software.

The results of the factor IXa stimulation by the FVIII-like activity exhibited by the IgM anti- FIX/FIXa-antibody 196/AF2 in generating actor Xa as judged by the readily measurable cleavage of the chromogenic substrate S-2222 (compare "16mU FVIII" and "196/AF2") is shown in Fig. 7A. Factor Xa activity is effectively blocked by the FXa specific inhibitor "Pefabloc Xa®" (compare "196/AF2" versus "196/AF2 35µM Pefabloc Xa®") indicating that indeed FXa was generated.

The same experiment was performed using purified IgG preparations of clone 198/AM1 (Fig. 7B). Purified IgG was diluted in TBS to a final concentration of

0,4mg/ml and 25µl (i.e. a total of 10µg), transferred to microtiter plate wells and warmed to 37°C. As a positive control, 6mU plasma- derived FVIII was used. 10µg unspecific mouse IgG (Sigma, I-5381) served as a negative control. The assay was performed as described above.

Further experiments show the factor IXa stimulation by the FVIII-like activity exhibited by the IgG anti-FIX/FIXa-antibody 198/AM1 generates factor Xa as judged by the readily measurable cleavage of the chromogenic substrate S-2222 (Fig. 7B). Again factor VIII and antibody 198/AM1 generate FXa which is effectively blocked by the FXa specific inhibitor "Pefabloc Xa®". As a negative control, unspecific mouse IgG (Sigma, I5381) was assayed.

In another set of experiments, the dependence of the FVIII-like activity of either purified anti-FIX/FIXa-antibodies (IgM, Fig.8A) or of unpurified antibodies derived from cell culture supernatants (IgG, Fig. 8B) on the presence of phospholipids (PL), FIXa/FX and Ca^{2+} was demonstrated. Mouse IgG was used as a control (Fig. 8C). Factor VIII-like activity was assayed essentially as described above. When indicated, either the FIXa/FX mixture, the PL or Ca^{2+} was omitted from the reaction. Absorbency at 405nm and 490nm of the samples was read at various times against a reagent blank (buffer instead of purified antibody) in a Labsystems iEMS Reader MF^{TM} microtiter plate reader. The results are shown in Fig. 8A, Fig. 8B and Fig. 8C.

The dependence of the FVIII-like activity of purified anti-FIXa-antibody 198/AC1/1 (IgG isotype, concentration used throughout the assay was 10µg/ml) on

the presence of phospholipids (PL), FIXa/FX and Ca^{2+} is further shown in Fig. 8A. As is easily recognizable, only the complete assay, including antibody, PL, Ca^{2+}, and FIXa/FX gives rise to a reasonable FXa generation. The dependence of the FVIII-like activity of cell culture supernatant containing unpurified IgM isotype anti-FIX/FIXa-antibody (196/AF1) on the presence of phospholipids, FIXa/FX and Ca^{2+} is shown in Fig. 8B.

Again, as already shown for the purified IgG preparation (Fig. 8A), antibody 198/AC1/1, only the complete assay, including PL, Ca^{2+}, FIXa/FX, will give a reasonable amount of FXa generation. To demonstrate the specificity of the reaction, total IgG prepared from normal mouse plasma was assayed under the same conditions as above. The results are shown in Fig. 8C. No FVIII-like activity could be detected. There is, as expected, no activity detectable in the absence of phospholipids, FIXa/FX and Ca^{2+}. All experiments were done in a microtiter plate and the OD405 was scanned every 5 minutes for 6h.

Example 6: Certain anti-FIX/FIXa-antibodies are procoagulant in the presence of FIXa

During normal hemostasis, FIX becomes initially activated either by the tissue factor (TF)/factor VIIa pathway or later on by activated factor XI (FXIa). Subsequent to its activation, FIXa associates on the platelet surface in a membrane bound complex with activated FVIII. Factor IXa by itself has little or no enzymatic activity towards FX, but becomes highly active in the presence of FVIIIa. To demonstrate that certain anti-FIX/FIXa antibodies have FVIII-like activity and hence are procoagulant in a FVIII deficient human plasma, the following experiment was carried out.

Different amounts of antibody 193/AD3 or mouse IgG (as a control) were used in a standard aPTT based one stage clotting assay. Briefly, 100µl of antibody-containing samples were incubated with 100µl of FVIII deficient plasma (DP) and with 100µl of DAPTTIN (PTT Reagent for determining activated Thromboplastin Time; IMMUNO AG) reagent, in a KC10A clotting analyzer. Where indicated, a total amount of 50ng activated FIX was included in the reaction mixture. After a 4 minute incubation, the reaction was started by the addition of 100µl $CaCl_2$ (25mM). The results are shown in Table 1 and Fig. 9.

	clotting time (sec)	
µg AB	193/AD3	mouse IgG
	50ng FIXa	50ngFIXa
9	101.6	102.5
4.5	95.6	103.2
2.25	93.1	103.2
1.8	93.7	101.9
1.35	91.4	103.4
0.9	94.4	102.2
0.45	98.1	101.9
0.34	97.1	103.9
0.23	99.3	103.7

Table 1: Clotting times of FVIII deficient plasma in an APTT based clotting assay employing various amounts of procoagulant (193/AD3) and control antibody (mouse IgG) in the presence of 50ng activated FIX (0.01UFIX). The molar ratio of antibody in the reaction and activated FIX is 10:1. The molar ratio between antibody and total FIX (FIX and FIXa, assuming that human FVIII deficient

plasma contains 1U (5µg) FIX) varies between 6:1 (9µg antibody in reaction) and 1:6 (0.23µg antibody in reaction). At the optimal shortening of the clotting time, the molar ratio between antibody and total FIX is 1:1. The clotting time without the addition of FIXa is in the range of 120 seconds.

Fig. 9 is a graphical representation of the clotting times of FVIII deficient plasma in an aPTT based clotting assay employing various amounts of procoagulant (193/AD3) and control (mouse IgG) antibody in the presence of 50ng activated FIX. There is a clear dose-dependent reduction of the clotting time in samples supplemented with antibody 193/AD3. These results imply that antibody 193/AD3 is procoagulant in the presence of FIXa.

Example 7: Anti-FIX/FIXa-antibodies are procoagulant in the presence of FVIII inhibitors and FIXa

A severe complication of the standard FVIII substitution therapy is the development of alloantibodies directed against FVIII, leading to FVIII neutralization and a condition where the patient's blood will not clot.

To demonstrate that certain anti-FIXa-antibodies have FVIII-like activity even in the presence of FVIII inhibitors, the following experiment was carried out. Different amounts of antibody 193/AD3 or, as a control, mouse IgG were used in a standard APTT based one-stage clotting assay. Briefly, 100µl antibody samples were incubated with either 100µl of FVIII deficient plasma (Fig.10A) or FVIII inhibitor plasma (inhibitor potency 400BU/ml), Fig.10B) as well as with 100µl of DAPTTIN

reagent, in a KC10A clotting analyzer. In addition, a total amount of 50ng activated FIXa was included in the reaction mixture. After a 4 minute incubation, the reaction was started by the addition of 100µl $CaCl_2$ (25mM). To ensure equal conditions, the experiments employing FVIII deficient plasma and FVIII inhibitor plasma were done side by side. The results are shown in Fig. 10A and 10B. As already shown in Example 6, there is a clear dose-dependent reduction of the clotting time in samples supplemented with antibody 193/AD3 in the presence of FVIII inhibitors.

Example 8: Anti-FIX/FIXa-antibodies are procoagulant in the presence of defective FVIII and FIXa

To demonstrate that certain anti-FIXa-antibodies have FVIII-like activity in the presence of defective FVIII, the following experiment may be carried out. Increasing amounts of antibody 193/AD3 or, as a control, mouse IgG are used in a standard aPTT-based one stage clotting assay. In this clotting assay, a hemophilia A patient's plasma having very low clotting activity due to the presence of defective FVIII (DF8) is used. Briefly, 100µl antibody samples are incubated with either 100µl of DF8 plasma or FVIII deficient plasma as well as with 100µl of DAPTTIN reagent, in a KC10A clotting analyzer. In addition, a total amount of 50ng activated FIXa is included in the reaction mixture. After a short incubation, the reaction will be started by the addition of 100µl $CaCl_2$ (25mM). To ensure equal conditions, the experiment employing FVIII deficient plasma and DF8 plasma is done side by side.

Example 9: Anti-FIX/FIXa-antibodies with procoagulant activity in the presence of FIXa distinguish between human and bovine FIXa

FIX/FIXa specific monoclonal antibodies selected from the 198th fusion experiment were purified from the respective hybridoma supernatant and quantified as described in Example 3. These antibodies were analyzed in a modified one-stage clotting assay (as described in Example 6) and some showed procoagulant activity.

The chromogenic activity of these antibody preparations was measured in the following FXa generation kinetic assay: 10µg of monoclonal antibody (in 25µl) were transferred to microtiter plate wells and warmed to 37°C. Chromogenic substrate (S-2222), synthetic thrombin inhibitor (I-2581), factor IXa and FX were reconstituted in sterile water and FIXa/FX (both bovine) were mixed with phospholipids according to the supplier's protocol. Per reaction, 50µl of the phospholipid/FIXa/FX solution were combined with 25µl $CaCl_2$ (25mM) and 50µl of the substrate/inhibitor cocktail. To start the reaction, 125µl of the premix were added to the monoclonal antibody solution in the microtiter plates and incubated at 37°C. Absorbance at 405nm and 490nm of the samples was read at various times (5min to 2h) against a reagent blank (25ml TBS instead of monoclonal antibodies) in a Labsystems iEMS Reader MF™ microtiter plate reader using GENESIS™ software. In parallel, the same reactions were performed except that 50ng human FIXa were added per reaction. Those antibodies that showed procoagulant activity had no chromogenic activity in the case of bovine FIX, but displayed high activity when human FIXa was present.

Fig. 11 shows the time course of the FVIII-like activity exhibited by the monoclonal antibodies 198/A1, 198/B1 and 198/AP1 with (+) and without (-) addition of 50ng human FIXaβ. Non-specific polyclonal mouse IgG was used as a control. 198/A1 and 198/B1 show procoagulant activity (similar as 193/AD3 in example 6) whereas 198/AP1 does not. Antibody 198/BB1 had the same activity pattern (data not shown).

Further monoclonal antibodies selected from the 198th fusion experiment include 198/D1 (ECACC NO. 99121616), 198/T2 (ECACC No. 99121617), 198/G2 (ECACC No.9912118), 198/U2 (ECACC No. 99121620).

Example 10 : Structure and procoagulant activity of antibody derivatives derived from anti-FIX/FIXa-antibodies; Subcloning antibody variable domains from hybridoma cell lines 193/AD3, 193/K2, 198/A1 and 198/B1 (clone AB2)

Cloning procedure: Messenger RNA was prepared from 1x10^6 hybridoma cells of the respective cell line (either 193/AD3, 193/K2, 198/A1 or 198/B1 (clone AB2)) employing the "QickPrep® Micro mRNA Purification Kit" (Pharmacia) according to the manufacturer's instructions. The corresponding cDNA was produced by retro transcription of mRNA using the "Ready-To-Go-You-Prime-First-Strand Beads kit" (Pharmacia) according to the manufacturer's instructions. Heavy and light chain encoding sequences were converted to the corresponding cDNA employing a set of primers. To reverse transcribe heavy chain-specific mRNA (VH), an equimolar mixture of the oligonucleotides MOCG1-2FOR (5' CTC AAT TTT CTT GTC CAC CTT GGT GC 3') (SEQ.ID.NO. 1), MOCG3FOR (5' CTC GAT TCT CTT GAT CAA CTC AGT CT 3') (SEQ.ID.NO. 2) and MOCMFOR (5' TGG AAT GGG CAC ATG CAG ATC TCT 3')

(SEQ.ID.NO. 3) was used (RTmix1). In the same reaction tube, light chain-specific cDNA (VL) was synthesized using primer MOCKFOR -(5' CTC ATT CCT GTT GAA GCT CTT GAC 3') (SEQ.ID.NO. 4).

The coding sequences for VH were amplified by PCR using the primer-sets depicted in Fig. 12 and the specific cDNA, derived from the reverse transcription mixture (RTmix1) described above, as the template. VK-chain genes were amplified using the primer sets depicted in Fig. 13 and also employing Rtmix1 as a template. The VH-PCR product was cleaved SfiI-AscI and inserted into SfiI-AscI digested vector pDAP2 (GeneBank accession no.: U35316). The pDAP2-VH constructs obtained thereby were named pDAP2-193AD3/VH, pDAP2-198A1/VH, pDAP2-198AB2/VH (derived from antibody 198/B1) and pDAP2-193/K2/VH, respectively. The plasmids were subsequently cleaved with AscI-NotI and the corresponding AscI-NotI digested VK-gene PCR product was inserted. The resultant vectors were designated pDAP2-193/AD3scFv, pDAP2-198/A1scFv, pDAP2-198/AB2scFv (derived from antibody 198/B1) and pDAP2-193/K2scFv and code for the VH-gene and the VL-gene of the monoclonal antibodies 193/AD3, 198/A1, 198/AB2 (derived from antibody 198/B1) and 193/K2. Heavy and light chains are linked by the coding sequence for an artificial, flexible linker ($G_4SGGRASG_4S$; Engelhardt et al., 1994) and enables expression of the scFv variant of the respective antibody.

In Fig. 14, the DNA and the deduced protein sequence of the scFv derived from the hybridoma cell line 193/AD3 are depicted. Nucleotides 1 to 357 code for the heavy chain variable domain, nucleotides 358 to 402 code for the artificial flexible linker and nucleotides

403 to 726 code for the light chain variable region. The protein sequence of the CDR3 region of the heavy chain has the sequence YGNSPKGFAY (SEQ.ID.NO. 5) and is given in bold letters. The artificial linker sequence ($G_4SGGRASG_4S$) is shown.

In Fig. 15, the DNA and the deduced protein sequence of the scFv derived from the hybridoma cell line 193/K2 is shown. Nucleotides 1 to 363 code for the heavy chain variable domain, nucleotides 364 to 408 code for the artificial flexible linker, and nucleotides 409 to 747 code for the light chain variable region. The protein sequence of the CDR3 of the heavy chain has the sequence DGGHGYGSSFDY (SEQ.ID.NO. 6); and is given in bold letters. The artificial linker sequence ($G_4SGGRASG_4S$) is show.

In Fig. 16, the DNA and the deduced protein sequence of the scFv derived from the hybridoma cell line 198/AB2 (derived from antibody 198/B1) are depicted. Nucleotides 1 to 366 code for the heavy chain variable domain, nucleotides 367 to 411 code for the artificial flexible linker, and nucleotides 412-747 code for the light chain variable region. The protein sequence of the CDR3 region of the heavy chain has the sequence EGGGFTVNWYFDV (SEQ.ID.NO. 7) and is given in bold letters. The artificial linker sequence ($G_4SGGRASG_4S$) is also shown.

In Fig. 17, the DNA and the deduced protein sequence of the scFv derived from the hybridoma cell line 198/A1 are depicted. Nucleotides 1 to 366 code for the heavy chain variable domain, nucleotides 367 to 411 code for an artificial flexible linker, and nucleotides 412-747 code for the light chain variable region. The protein sequence of the CDR3 region of the heavy chain has the

sequence EGGGYYVNWYFDV (SEQ.ID.NO.8) and is given in bold letters. The artificial linker sequence ($G_4SGGRASG_4S$) is also shown.

Example 11: Procoagulant activity of peptides derived from CDR3 regions of anti-FIX/FIXa-antibodies

In principle, the antibody molecule can be envisioned as a biological device for the presentation of a combinatorial array of peptide elements in three dimensional space (see Gao et al., 1999, PNAS, 96:6025). Therefore, an antibody (or an antibody derivative, e.g. scFv, Fab, etc.) can be used either as a tool for the detection of functionally important domains of a specific target protein, or on the other hand, for the delineation of amino acid sequences specifically mediating certain interactions, i.e. activating or enhancing the activity of FIXa towards the physiological substrate FX. The latter process has led to the evaluation of a number of heavy chain CDR3 region ($CDR3_H$) derived peptide sequences as FIXa enhancing agents.

Enhancing the procoagulant activity of peptides which exhibit such activity may be accomplished through sequence variation within the peptide regions critical for mediating the FIXa activity enhancement. As a possible step towards peptide sequences with enhanced procoagulant activity, the binding site of an antibody, i.e. 198/A1 or 198/B1, on the FIXa molecule is mapped by employing sequence comparison analyses, competitive binding assays, Western blot analyses and competitive ELISA analyses. Since the crystal structure of FIX is known, molecular modeling is subsequently used to

improve the fitting of i.e. 198/B1 derived peptides in the 198/B1 binding site on human FIXa.

On the other hand, methodical mutational analysis of a given peptide sequence such as 198/A1 or 198/B1 $CDR3_H$ derived peptide sequences by, e.g., "alanine scanning mutational analysis" allows for the identification of peptide residues critical for procoagulant activity. Another way to improve the activity of a certain peptide sequence is the use of peptide libraries combined with high throughput screening.

The antigen binding site of an antibody is derived from the juxtaposition of the six "complement determining regions (CDR's)" at the N-terminal end of the VL-HL dimer (or Fv region). The contribution of a single CDR to the antibody specificity for a given antigen may vary considerably, but in general it is thought that the CDR3 region of the heavy chain ($CDR3_H$) is of special influence, i.e. the particular protein sequence of $CDR3_H$ region may be highly important for antigen recognition. The length of $CDR3_H$ regions has been reported to vary considerably and is in the range of 4-25 amino acids (Borrebaeck, p.16).

An example of a methodical mutational analysis of peptide sequences is given below. To improve the solubility/procoagulant efficacy of peptides derived from the CD3-region of anti FIX/FIXa antibodies, the N-terminal as well as the C-terminal amino acid sequences were changed. In addition, a series of mutated peptides was constructed and analyzed.

The principle of such a study is exemplified by a series of peptides derived from $CDR3_H$ region of antibodies 198/A1 and 198/B1. The original peptide A1(see table

2)is derived from the $CDR3_H$ region of antibody 198/A1 and peptide B1 is derived from the $CDR3_H$ region of antibody 198/B1, respectively (see example 10, Fig. 16 and 17). The term "scrambled version"means that a peptide has the same amino acids but in random order.

Peptide	Sequence	Amino-acids	MW (D)	pI	Remark
A1	EGGGYYVNWYFDV (SEQ.ID.No. 9)	(13aa)	1569	7,2	Decreased solubility
A1/1	VYGFGWGYEVNDY (SEQ.ID.No. 10)	(13aa)	1569	7,1	Scrambled version of A1,
A1/2	EEEEGGGYYVNWYFDEEE (SEQ.ID.No. 11)	(18aa)	2244	5,8	Acidic pI, soluble,
A1/3	RRREGGGYYVNWYFDRRR (SEQ.ID.No. 12)	(18aa)	2407	9,9	Basic pI, soluble,
A1/4	EYGEGYGEVNEYDEFEWE (SEQ.ID.No. 13)	(18aa)	2244	5,8	Scrambled version of A1/2
A1/5	VRYRNRYRWGYRGRFGDE (SEQ.ID.No. 14)	(18aa)	2407	9,9	Scrambled version of A1/3
A1/3-scr3	RRRGEYGVYWNGDFYRRR (SEQ.ID.No. 15)	(18aa)	2407	9,9	Scrambled version of A1/3
A1/3-Rd	RdRdRdEGGGYYVNWYFDRdRdRd (SEQ.ID.No. 16)	(18aa)	2407	9,9	Peptide A1/3 but substitute D-Arg for L-Arg
A1/3-Rd-srmb	RdRdRdGEYGVYWNGDFYRdRdRd (SEQ.ID.No. 17)	(18aa)	2407	9,9	Scrambled version of A1/3-Rd

Table 2

List of a series of antibody 198/A1 derived peptides. Listed are the length of the peptide (aa, amino acids #), the calculated molecular weight (MW, in Dalton (D) and the statistical isoelectric point (pI).D-Arg is

abbreviated as Rd.

In a first series of experiments we improved the solubility of the original $CDR3_H$ peptide sequence (A1; EGGGYYVNWYFDV) by removing the C-terminal Val residue and adding several charged residues at the N- as well as the C-terminal end of the peptide. The resulting peptides, A1/2 (acidic pI), A1/3 (basic pI) and their respective scrambled versions A1/4, A1/5 and A1/3scr3 were readily soluble in a variety of buffer systems at physiological pH.

To analyze the FVIII-like (FIXa activating) activity of the peptides, an assay system based on a commercial available FVIII assay was developed (see examples 2 and 4). The basic principle is, that without a cofactor, FIXa will have very limited activity towards its natural substrate FX. Only in the presence of a substance having FIXa activation properties, i.e. FVIII or a substance exhibiting FVIII-like activity, a substantial amount of FXa is produced by cleavage of FX through the FIXa/activator complex. The amount of FXa generated is monitored by cleavage of a chromogenic substrate. The principle of the revised chromogenic assay is described for two representative peptides: A1/3 and A1/5 (Table 2). Briefly, 25µl aliquots of peptide stock solution (in imidazole buffer (IZ) 50mM imidazole, 100mM NaCl, pH7.2) were transferred to microtiter plate wells and warmed to 37°C. Chromogenic FXa substrate (S-2222), synthetic thrombin inhibitor (I-2581), bovine FIXa and bovine FX were reconstituted in sterile water and FIXa/FX mixed with phospholipids according to the supplier's protocol.

Since the peptides do not react with bovine FIXa, (which comes as a mixture with bovine FX in the Test Kit) 2,9nM (in most cases 2.3nM) human FIXa (ERL) were added (see Example 11, Fig 19). Per reaction, 50µl of the phospholipid /FIXa/FX solution were combined with 25µl $CaCl_2$ (25mM) and 50µl of the substrate/inhibitor cocktail. To start the reaction, 125µl of the premix were added to the peptide solution in the microtiter plate and incubated at 37°C. Absorbance at 405nm and 490nm of the samples was read at various times (5 min to 2h) against a reagent blank in a Labsystems iEMS Reader MF™ microtiter plate reader using GENESIS™ software. The result of this experiment are shown in Example 11, Fig 18. Peptide A1/3 induced a readily measurable FXa generation in the presence of 2.9nM human FIXa, whereas the scrambled version A1/5 was inactive. In addition, the acidic peptide A1/2 as well as the scrambled versions A1/4 and A1/3-scr3 did not give any significant chromogenic activity when tested under comparable conditions (data not shown). To prove that the peptide A1/3 like the parental antibody 198/A1 does not react with bovine FIXa and FX the experiment shown in Fig. 19 was done. The peptide A1/3 was incubated as described above with (A1/3 (24µM), +hFIXa) and without (A1/3 (24µM), w/o hFIXa) 2.3nM human FIXa (hFIXa). In a control experiment we added plain dilution buffer (IZ) supplemented with 2.3nM hFIXa to the reaction mixture. As shown in Fig. 19, the reaction takes place only in the presence of human FIXa.

Fig. 18 demonstrates the chromogenic FVIII-like activity of peptide A1/3 in the presence of 2.9nM human FIXa (hFIXa). The scrambled version of peptide A1/3, peptide

A1/5 does not give rise to any FXa generation.

Fig. 19 demonstrates the dependence of the chromogenic FVIII-like activity of peptide A1/3 on the presence of human FIXa (hFIXa). In the absence of human FIXa, peptide A1/3 does not give rise to any FXa generation. The buffer control, plain imidazole buffer is designated IZ.

The peptides were also analyzed for their potential to reduce the clotting time in a FVIII deficient plasma. The aPTT based one stage clotting assay was essentially done as described (see example 6). Clotting times (time from starting the reaction to the "clot"-formation were compared either against FVIII, a buffer control (IZ) or a control peptide (scrambled version). The results of two typical clotting experiments done with two different aPTT reagents (DAPTTIN and Pathromtin SL) are shown in table 3A and table 3B.

Exp. 1	peptide conc.	w/o FIXa sec	w/o FIXa sec	**average sec**	2.2nM FIXa sec	2.2nM FIXa sec	**average sec**
IZ	0	107,7	106,8	**107**	93,1	94,5	**94**
A1/3	15µM	78,2	77,1	**78**	59,3	59,9	**60**
	12,5µM	80,2	80,6	**80**	60,2	58,9	**60**
	7,5µM	97,8	97,9	**98**	73,1	72,7	**73**
	2,5µM	105,2	104,8	**105**	91,1	91	**91**
A1/3 - scr3	15µM	122,5	122	**122**	106,1	105,5	**106**
	12,5µM	116	117,6	**117**	103,1	104,5	**104**
	7,5µM	114,2	113,9	**114**	100,8	100,6	**101**
	2,5µM	107,8	107,4	**108**	96,3	95,2	**96**
Exp. 2	peptide conc.	w/o FIXa sec	w/o FIXa sec	**average (sec)**	2.2nM FIXa sec	2.2nM FIXa sec	**average (sec)**
IZ	0	111	109,7	**110**	94,7	95,5	**95**
A1/3	12.5µM	83,6	85,5	**85**	56,7	56,7	**57**
	10µM	79,1	78,5	**79**	63,1	62,5	**63**
	7.5µM	100,1	100,5	**100**	71,6	73,9	**73**
	5µM	103,4	104,8	**104**	77	76	**77**
	2.5µM	110,1	108,9	**110**	88	88,8	**88**
	1,25µM	108,7	109,3	**109**	90,7	90,8	**91**

Table 3A. Clotting activity of peptides A1/3 and A1/3-scr (scrambled version of A1/3) in FVIII deficient plasma either in the presence or in the absence (w/o) of 2.2nM human FIXa. Shown are two independent representative experiments (Exp. 1 and Exp. 2). All clotting experiments have been done in duplicate. Given are the clotting times for the individual experiments and the average clotting time in seconds (sec). Experiments shown in table 3A have been done employing the aPTT reagent DAPTTIN (Baxter Hyland Immuno). Compared to the buffer control (IZ, imidazole buffer) the peptide A1/3 gave rise to a dose dependent reduction in the clotting time. The reduction in the clotting time

became much more pronounced by the addition of 2.2nM activated human FIX to the reaction mix. The scrambled version of peptide A1/3, A1/3-scr3 did not show any reduction of the clotting time. In fact, at concentrations above 2.5µM, the scrambled peptide became inhibitory and therefore prolonged the clotting time. Peptides A1/1, A1/2, A1/4 and A1/5 did not give any reduction in the clotting time indicating that they lack procoagulant activity (data not shown).

	Final conc.	w/o FIXa sec	**w/o FIXa sec**	average sec	2.2nM FIXa sec	2.2nM FIXa sec	**average sec**
IZ	0	131,8	**132,1**	132	107,9	108,7	**108**
FVIII	12,5mU/ml	68,9	**69**	69	52,9	53,6	**53**
	6,25mU/ml	77,8	**77,9**	78	58,6	58,9	**59**
A1/3							
	15µM	152,8	**149,3**	151	75,4	75,2	**75**
	10µM	135,7	**134,6**	135	76,2	79,8	**78**
	5µM	152,6	**155,6**	154	86,6	90,2	**88**
	1µM	138,3	**138,8**	139	103,7	105,9	**105**

Table 3B. Clotting activity of peptide A1/3 in FVIII deficient plasma when Pathromtin SL (DADE Behring)is used as an aPTT reagent. The experiments were done in duplicate, either in the presence or in the absence (w/o) of 2.2nM human FIXa. Given are the clotting times for the individual experiments and the average clotting time in seconds (sec). Factor VIII and imidazole buffer (IZ) were included as positive and negative control

respectively.

In contrast to the experiments shown in table 3A the experiments shown in table 3B have been done employing the aPTT reagent Pathromtin SL. In the presence of FIXa, the peptide A1/3 gave rise to a dose dependent reduction in the clotting time whereas in the absence of FIXa no reduction of the clotting time was detectable.

In another series of experiments we set out to improve the plasma stability (protection from, e.g., proteolytic degradation) of peptide A1/3. One approach was to substitute the N- and C-terminal L-Arg residues with D-Arg residues (exemplified by peptides A1/3-rd and A1/3-Rd-srmb). Peptides A1/3-rd and A1/3-Rd-srmb (scrambled version of the peptide)were then analyzed in a chromogenic as well as in the aPTT based clotting assay. These experiments revealed that exchanging the terminal L-Arg residues for D-Arg residues did not change the FVIII-like activity as measured in the chromogenic assay, indicating that chirality of the Arg-residues does not play a major role in chromogenic activity (Fig. 20). In addition, the aPTT based one-stage clotting activity, although somewhat reduced, was still easily detectable (Table 4).

	Peptide conc.	w/o FIXa sec	w/o FIXa, sec	**average sec**	2.2nM FIXa sec	2.2nM FIXa sec	**average sec**
IZ	0	110	109,1	**110**	96	96	96
A1/3	15µM	77,8	78	**78**	56,1	55,5	56
	12,5µM	99,4	100,5	**100**	65	68	67
	10µM	104,4	104,5	**104**	72	73,2	73
	7,5µM	105,2	105,2	**105**	80,7	80,5	81
	5µM	108,4	107,7	**108**	89,7	88,3	89
	2,5µM	107,9	107,6	**108**	93,6	93,3	93
	1,25µM	106,7	107	**107**	94,4	95	95
A1/3-Rd	15µM	96,4	95,4	**96**	76,1	74,4	75
	12,5µM	98	98,6	**98**	72,3	73,7	73
	10µM	93,5	95,8	**95**	74,2	77,2	76
	7,5µM	97,6	98,1	**98**	80,9	82,2	82
	5µM	99,2	99,1	**99**	86	85,1	86
	2,5µM	102,7	103,4	**103**	94,4	94,7	95
	1,25µM	107,5	107,7	**108**	96,6	96	96
A1/3-Rd srmb	15µM	121,9	121,3	**122**	112,7	112,4	113
	12,5µM	117,2	118	**118**	108,1	107,8	108
	10µM	115,8	115,3	**116**	107,2	107,8	108
	7,5µM	114,6	113,6	**114**	107,6	106,6	107
	5µM	113,1	112,4	**113**	108,5	108,2	108
	2,5µM	111,9	111,9	**112**	105	104,2	105
	1,25µM	107,2	107,1	**107**	101,1	105,3	103

Table 4 One stage clotting activity of peptides A1/3, A1/3-Rd and A1/3-Rd-srmb (sequences see table 2). IZ, buffer control.

Fig. 20 demonstrates the unchanged chromogenic activity of peptide A1/3-Rd. Peptides at a final concentration of 12µM or the buffer control (IZ) were incubated in the presence of 2.3nM human FIXa (+). The chromogenic activity of peptide A1/3 and A1/3-Rd was found to be virtually unchanged and gave almost identical results in the chromogenic assay. The scrambled version of peptide A1/3, A1/5 as well as the buffer gave no significant FXa

generation.

In the next series of experiments we set out to determine the individual role of any amino acid of the peptide core sequence by substituting each residue for the amino acid Alanine (Table 5).

Peptide	Sequence	Amino acid #	MW (D)	pI	Remark
A1/3	RRREGGGYYVNWYFDRRR (SEQ.ID.No. 18)	(18aa)	2407	9,9	Basic pI, soluble,
A1/3-13	RRR**A**GGGYYVNWYFDRRR (SEQ.ID.No. 19)	(18aa)	2349	10.4	E_1-A_1
A1/3-1	RRRE**A**GGYYVNWYFDRRR (SEQ.ID.No. 20)	(18aa)	2421	9.9	G_2-A_2
A1/3-2	RRREG**A**GYYVNWYFDRRR (SEQ.ID.No. 21)	(18aa)	2421	9.9	G_3-A_3
A1/3-3	RRREGG**A**YYVNWYFDRRR (SEQ.ID.No. 22)	(18aa)	2421	9.9	G_4-A_4
A1/3-4	RRREGGG**A**YVNWYFDRRR (SEQ.ID.No. 23)	(18aa)	2315	9.9	Y_5-A_5
A1/3-5	RRREGGGY**A**VNWYFDRRR (SEQ.ID.No. 24)	(18aa)	2315	9.9	Y_6-A_6
A1/3-6	RRREGGGYY**A**NWYFDRRR (SEQ.ID.No. 25)	(18aa)	2379	9.9	V_7-A_7
A1/3-7	RRREGGGYYV**A**WYFDRRR (SEQ.ID.No. 26)	(18aa)	2364 .	9.9	N_8-A_8
A1/3-8	RRREGGGYYVN**A**YFDRRR (SEQ.ID.No. 27)	(18aa)	2292	9.9	W_9-A_9
A1/3-9	RRREGGGYYVNW**A**FDRRR (SEQ.ID.No. 28)	(18aa)	2315	9.9	Y_{10}-A_{10}
A1/3-10	RRREGGGYYVNWY**A**DRRR (SEQ.ID.No. 29)	(18aa)	2331	9.9	F_{11}-A_{11}
A1/3-11	RRREGGGYYVNWYF**A**RRR (SEQ.ID.No. 30)	(18aa)	2363	10.5	D_{12}-A_{12}
A1/3-12srmb	RRRYVYNGWGYFEG**A**RRR (SEQ.ID.No. 31)	(18aa)	2363	10.4	Scrambled version

Table 5. Listed are the peptides designed to elucidate the role of any single amino acid within the peptide core sequence ($E_1G_2G_3G_4Y_5Y_6V_7N_8W_9Y_{10}F_{11}D_{12}$). The lower case numbers describe the position of the amino acid within the peptide. Alanine, an uncharged small amino acid, was substituted for each amino acid ("Alanine scan"). Also listed are the lengths of the peptides (amino acids #), the calculated molecular weights (MW, in Dalton (D) and the statistical isoelectric points (pI).

Each of the peptides was dissolved individually in imidazole buffer (50mM imidazole, 100mM NaCl, pH7.2) and subsequently diluted in clotting buffer (50mM imidazole, 100mM NaCl, 1% human albumin, pH7.4) to the desired final concentration. The peptides were analyzed for their chromogenic activity as well as for their potential to reduce the clotting time in a FVIII deficient plasma. The one-stage clotting assay was essentially done as described (see example 6). Clotting times (time from starting the reaction to the "clot"-formation were compared either against a buffer control or a control peptide (scrambled version).

Some of the results of the "Alanine scan" are given for the peptides A1/3-2 and A1/3-3. The change of G_3-A_3 as exemplified in the peptide A1/3-2 yields high chromogenic activity and a strong reduction of the one-stage clotting time (34 seconds at a concentration of 12.5µM) in the presence of 2.2nM human FIXa. Peptide A1/3-3 (G_4-A_4) exhibits an optimum of chromogenic activity around a final concentration of 12µM with decreased activity at either higher or lower

concentrations. The peptide is somewhat inhibitory in a one-stage clotting assay at higher concentrations (12.5μM) in the absence of FIXa but becomes strongly active in the presence of 2.2nM FIXa (31 seconds, 12.5μM).

In the next series of experiments we set out to determine the individual role of any amino acid of the peptide core sequence by substituting each core residue for the amino acid glutamic acid (E) (see Table 6).

Peptide	Sequence	Amino-Acids	MW (D)	pI	Remark
A1/3	RRREGGGYYVNWYFDRRR	(18aa)	2407	9;9	Basic pI, soluble,
A1/3-22	RRRE**E**GGYYVNWYFDRRR (SEQ.ID.No. 32)	(18aa)	2479	9.5	G_2-E_2
A1/3-23	RRREG**E**GYYVNWYFDRRR (SEQ.ID.No. 33)	(18aa)	2479	9.5	G_3-E_3
A1/3-24	RRREGG**E**YYVNWYFDRRR (SEQ.ID.No. 34)	(18aa)	2479	9.5	G_4-E_4
A1/3-26	RRREGGG**E**YVNWYFDRRR (SEQ.ID.No. 35)	(18aa)	2373	9.4	Y_5-E_5
A1/3-27	RRREGGGY**E**VNWYFDRRR (SEQ.ID.No. 36)	(18aa)	2373	9.4	Y_6-E_6
A1/3-28	RRREGGGYY**E**NWYFDRRR (SEQ.ID.No. 37)	(18aa)	2437	9.5	V_7-E_7
A1/3-29	RRREGGGYYV**E**WYFDRRR (SEQ.ID.No. 38)	(18aa)	2422	9.5	N_8-E_8
A1/3-30	RRREGGGYYVN**E**YFDRRR (SEQ.ID.No. 39)	(18aa)	2350	9.5	W_9-E_9
A1/3-31	RRREGGGYYVNW**E**FDRRR (SEQ.ID.No. 40)	(18aa)	2373	9.4	Y_{10}-E_{10}
A1/3-32	RRREGGGYYVNWY**E**DRRR (SEQ.ID.No. 41)	(18aa)	2389	9.5	F_{11}-E_{11}
A1/3-33	RRREGGGYYVNWYF**E**RRR (SEQ.ID.No. 42)	(18aa)	2421	9.9	D_{12}-E_{12}
A1/3-34srmb	RRRGEYGEYWNGDFYRRR (SEQ.ID.No. 43)	(18aa)	2437	9.5	Scrambled version

Table 6. Listed are the peptides designed to elucidate

the role of any single amino acid within the peptide core sequence ($E_1G_2G_3G_4Y_5Y_6V_7N_8W_9Y_{10}F_{11}D_{12}$). The lower case numbers describe the position of the amino acid within the peptide. Glutamic acid, a negatively charged large amino acid, was substituted for each amino acid of the core sequence ("Glutamic acid scan"). Also listed are the lengths of the peptide (amino acids #), the calculated molecular weights (MW, in Dalton (D) and the statistical isoelectric points (pI).

Each of the peptides was solved individually in imodazole buffer (50mM imidazole, 100mM NaCl, pH7.2) and subsequently diluted in clotting buffer (50mM imidazole, 100mM NaCl, 1% human albumin, pH7.4) to the desired final concentration. The peptides derived from the "Glutamic acid scan" series were analyzed for their chromogenic FVIII-like activity as well as for their potential to reduce the clotting time in a FVIII deficient plasma. The one-stage clotting assay was essentially done as described (see example 6).

The peptide A1/3-24 showed some interesting properties. The molecule exhibited high chromogenic FVIII-like activity at concentrations between 6.5µM-12µM but lost activity at higher concentrations (up to 24µM). The peptide had no procoagulant activity in the absence of human FIXa but was strongly active in the presence of 2.2nM hFIXa.

In a second series of experiments we set out to improve the procoagulant activity of the antibody 198/B1 CDR3H derived peptide sequence B1. In a first step we improved the solubility of the original peptide sequence (B1; EGGGFTVNWYFDV) by removing the C-terminal Val residue

and adding several charged residues at the N- as well as the C-terminal end of the peptide. The resulting peptides B1/4, B1/6 (acidic pI), B1/7 (basic pI) and their scrambled versions B1/5, B1/7scr3 are readily soluble in a variety of buffer systems at physiological pH.

Peptide	Sequence	Amino-acids	MW (D)	pI	Remark
B1	EGGGFTVNWYFDV (SEQ.ID.No. 44)	(13aa)	1491	6,0	Decreased solubility
B1/4	REGGGFTVNWYFDR (SEQ.ID.No. 45)	(14aa)	1704	7,9	Soluble,
B1/5	FGVGYRGETRNFDW (SEQ:ID:No. 46)	(14aa)	1704	8,0	Scrambled version, soluble
B1/6	EEEEGGGFTVNWYFDEEE (SEQ.ID.No. 47)	(18aa)	2166	5,0	Acidic pI soluble
B1/7	RRREGGGFTVNWYFDRRR (SEQ.ID.No. 48)	(18aa)	2329	9,9	Basic pI soluble
B1/7scr3	RRRFGVGYGETNFDWRRR (SEQ.ID.No. 49)	(18aa)	2329	9,9	Basic pI, soluble, scrambled version

Table 7 is a list of a series of antibody 198/B1 derived peptides. Listed are the length of the peptide (aa, amino acids #), the calculated molecular weight (MW, in Dalton (D) and the statistical isoelectric point (pI)..

Peptides B1/4 and B1/5 were soluble in 50mM Tris, 100mM NaCl, pH=6.5. Both peptides were analyzed in a chromogenic FVIII assay. Peptide B1/4 but not the scrambled version B1/5 was found to have some chromogenic activity (data not shown).

Subsequently peptides B1/6, B1/7 and B1/7scr3 were analyzed. Each of the peptides was solved individually

in 50mM imidazole, 100mM NaCl, pH7.2 and subsequently diluted either in clotting buffer (50mM imidazole, 100mM NaCl, 1% human albumin, pH7.4) or in imidazole buffer to the desired final concentration. The peptides were analyzed for their chromogenic activity as well as for their potential to reduce the clotting time in a FVIII deficient plasma (table 8 & 9). The one stage clotting assay was essentially done as described (see example 6). Clotting times (time from starting the reaction to the "clot"-formation were compared either against a buffer control or a control peptide (scrambled version).

The FIXa activating activity (FVIII cofactor-like activity) from peptide B1/7 was first measured in the chromogenic assay described above.

As shown in Fig. 21, the addition of 2.4µM peptide B1/7 to the reaction mixture led to a well measurable generation of FXa. In contrast, the addition of 35µM Pefabloc Xa, a specific inhibitor of FXa protease activity, resulted in a significant reduction of the chromogenic substrate cleavage reaction (Fig. 22) thereby proving that there was indeed a peptide-FIXa mediated FXa generation. If there was no addition of FIXa and FX to the reaction mixture, no FXa was synthesized (Fig. 22). Peptide B1/6 and the control peptides B1/5 and B1/7scr3 exhibited no activity (data not shown).

Fig. 21 demonstrates the chromogenic activity of peptide B1/7. The peptide at a final concentration of 2.4µM or the buffer control (IZ) were incubated in the presence of 2.3nM human FIXa.

In Fig. 22 peptide B1/7 at a final concentration of 2.4µM or the buffer control (IZ) were incubated in the

presence of 2.3nM human FIXa (as indicated either as"+2.3nM hFIXa" or "+"). The chromogenic activity of peptide B1/7 was found to be dependent on the presence of FIXa and FX since no reaction is detectable when FIXa and FX are left out of the reaction (w/o FIXa/FX). To prove that the peptide B1/7 mediates indeed FXa generation, the FXa specific protease inhibitor Pefabloc Xa was added to the reaction mix (35µM Pefabloc Xa).

In a second set of experiments, the procoagulant effect of peptides B1/6, B1/7 and B1/7scr3 were tested in a aPTT based one-step coagulation assay. The experiments were done essentially as described in Example 6. The results are shown in tables 8 and 9.

Peptide	12,5µM (-)	1.25µM (-)	0.125µM (-)	12,5nM (-)	Buffer (-)	remarks
B1/6	115	110	111	111	110	
B1/7	157	112	109	110	110	
B1/7scr3	115	105	106	105	107	

Table 8: FVIII deficient plasma was incubated either with peptides B1/6, B1/7scr3 or B1/7 in the absence of activated human FIX. As a negative control, plain buffer was added to the deficient plasma. The clotting times for the various combinations are given. Under these conditions, peptide B1/7 at its highest concentration (12.5µM) becomes inhibitory to the coagulation process as indicated by the extended clotting time of 157 seconds.

Peptide	12,5µM (+)	1.25µM (+)	0.125µM (+)	12,5nM (+)	Buffer (+)	remarks
B1/6	103	100	101	100	100	
B1/7	83	92	99	99	100	
B1/7scr3	102	94	94	94	94	

Table 9: FVIII deficient plasma was incubated either with peptides B1/6, B1/7scr3 or B1/7 in the presence of activated human FIX. As a negative control, plain buffer was added to the deficient plasma. The clotting times for the various combinations are given. In the presence of FIXa, peptide B1/7 becomes procoagulant as indicated by the reduced clotting time (83 seconds compared to 102 seconds for the scrambled peptide and 100 seconds for the buffer control)

Example 12: Procoagulant activity of peptide derivatives obtained from CDR3 regions of anti- FIX/FIXa-antibodies in FVIII inhibitor plasma

To assay for the procoagulant activity of peptide A1/3 in FVIII inhibitor plasma the following experiment was carried out. We performed a standard aPTT based one stage clotting assay, but instead of FVIII deficient plasma we employed FVIII inhibitor plasma. The inhibitory potency of the plasma was 8.1 Bethesda Units per ml.

		w/o FIXa	w/o FIXa			FIXa	FIXa	
	Peptide conc.	sec	sec	**Average sec**		sec	sec	**average sec**
IZ	0	104,8	103,6	**104**		94,2	94,1	**94**
A1/3	12,5µM	85,8	85,3	**86**		61	60,2	**61**
	10µM	88,4	87,9	**88**		61,3	61,8	**62**
	7,5µM	93,7	92,7	**93**		68,8	70,9	**70**
	5µM	101,5	101,1	**101**		81	82	**82**
	2,5µM	106,1	105,3	**106**		90,2	90,5	**90**
	1,25µM	104,5	104,3	**104**		91,3	91,4	**91**

Table 10: Various amounts of peptide A1/3 (12.5µM-1.25µM) were added to FVIII inhibitor plasma (either in the presence (FIXa) of 2.2nM FIXa or in the absence (w/o FIXa). As a negative control, plain buffer was added to the plasma (IZ). Experiments were done in duplicate and the average (aver.) was calculated. The clotting times (in seconds) for the various combinations are given. It is easily appreciable that the peptide A1/3 reduces (in a dose dependent manner) the clotting time of FVIII inhibitor plasma in the presence of FIXa but, although albeit to a much lesser extent, also in the absence of

FIXa.

Example 13: Conversion of the 196/C4 IgM into IgG1

Since some IgM antibodies demonstrate high FVIII-like activity in chromogenic assays, attempts were made to convert such IgM antibodies into IgG antibodies (though antibody derivatives such as Fab, F(ab)2, scFv, etc. could also be produced). Described in detail below is the rescue of the IgM variable region genes.

Expression vector pBax-IgG1 (Fig. 23) was first constructed from vectors pSI (Promega) and pEF/Bsd (Invitrogen) through multiple cloning steps. B-lymphocytes of a donor are purified from blood and mature mRNA purified from these cells using the "micro-mRNA purification-kit" (Pharmacia). The cDNA of a human kappa chain and a human gamma 1 chain are prepared employing the "you-primefirst-strand-cDNA-"kit" (Pharmacia) using specific primers.

The coding sequence of a human kappa light chain constant domain is amplified from the cDNA by PCR using specific primers.

The gene of a human gamma 1 chain constant region (CH1-hinge-CH2-CH3) is amplified from the cDNA by PCR using specific primers.

The PCR product of the light chain constant domain is digested with XbaI and NheI and inserted into digested pSI. The resultant vector is cleaved with EcoRI and XbaI and annealed oligonucleotides are inserted, resulting in vector pSI-Ckappa. The annealed oligonucleotides provide for the leader and the SacI-XbaI sites for insertion of the kappa chain variable region. The PCR product of the human gamma 1 chain constant region is digested with SpeI and BamHI and inserted into digested pSI. The resultant vector is

cleaved with SpeI and NotI and annealed oligonucleotides are inserted resulting in vector pSI-Cgamma. The annealed oligonucleotides provide for the leader and the XhoI-BstEI sites for insertion of the heavy chain variable region. Vector pEF/Bsd is digested NheI and SfiI, blunt ended by Klenow treatment and the whole expression cassette of pSI-Ckappa, excised with BglII and BamH1, is inserted (after Klenow treatment). The resultant vector is digested with EcoRI and HindIII and treated with Klenow. The whole expression cassette of pSI-Cgamma is excised with BglII and BamH1 and is inserted (after Klenow treatment). The resultant vector is named pBax-IgG1.

The light chain variable region can be inserted in between the SacI-XbaI sites, yielding the complete coding-sequence of a kappa light chain. The heavy chain variable region can be cloned in between the XhoI-BstEI sites, resulting in a complete IgG1 heavy chain gene. Both open reading frames are expressed under the control of the SV40-promoter and harbour the coding sequence of a signal peptide at the 5' end of the genes for secretion of the heavy and light chains into the endoplasmatic reticulum. Transfection into COS cells allows the expression of an IgG1 with the same binding properties as the parental IgM.

Construction of the plasmid pBax-196/C4 is further accomplished by amplifying the VH of the 196/C4 scFv (subcloned as described in Experiment 10) by PCR using specific primers. The PCR product is digested with XhoI and BstEII and inserted into XhoI and BstEII digested pBax IgG1. The VL of the 196/C4 scFv is amplified by PCR using specific primers. The PCR product is digested with SacI and XbaI and inserted into SacI and XbaI-digested

pBax IgG1-VH. The resultant vector (pBax-196/C4) is transfected into COS cells by electroporation, and hybrid IgG1 molecules (murine variable region and human constant region) with the same specificity as the parental IgM is expressed.

Example 14: Activation of FIXa amydolytic activity by anti-FIXa antibodies:

Briefly, 20µl factor IXa (containing 200mU FIXa (Stago)) were incubated at 37°C, with 200µl of reaction buffer (50mM TrisHCl pH7.4, 100mM NaCl, 5mM $CaCl_2$ and 40% Ethyleneglycol), 25µl of FIXa substrate (CH_3SO_2-D-CHG-Gly-Arg-pNA,AcOH, 10µM/ml, Pentapharm LTD) in the absence or presence of various amounts of anti-FIX antibodies 198/B1 (IgG isotype) or 196/AF1 (IgM isotype). Specific cleavage of FIXa substrate was monitored at 405nm in an ELISA reader.

The presence of the anti-FIX antibodies enhanced the amydolytic activity of FIXa at least 2 fold.

Fig. 24 shows the increase of the amidolytic activity of FIXa in the presence of antibody 198/B1 (Fig. 24A) and antibody 198/AF1 (Fig. 24B).

Example 15: FVIII-like activity exhibited by Fab fragments derived from anti- FIX/FIXa-antibodies.

Fab fragments of anti-FIX/FIXa antibodies were prepared and purified according to standard protocols. Briefly, 1ml antibody 198/A1(4mg/ml in 50mM imidazole, 100mM NaCl, pH7.4) was incubated overnight with 87µl fragmentation buffer (1M Na Acetate, 10mM EDTA 67.5mg/ml L-cysteine) and 0.25mg papain (immobilized on agarose beads), at 37°C. The preparation was filtered to remove the papain. L-histidine was added (final concentration 50mM) and afterwards the pH was adjusted to 7.0. Finally, solid NaCl is added to give a final

concentration of 1M.

Subsequently, the 198/A1 Fab fragment was purified by binding to protein L: We used ImmunoPure Immobilized PROTEIN L Plus (Pierce) in a PHARMACIA XK 16/20 Column (gel-volume: 2ml) Buffers for chromatography were: 1) equilibration-buffer : 50mM L-histidine pH 7.0; 1M NaCl; 0,1% (w/v) NaN_3; 2) wash-buffer: 50mM L-Histidine pH 7.0; 0.1% (w/v) NaN_3; 3)elution-buffer: 100 mM glycine pH 2.5; 0.1% (w/v) NaN_3; and 4) neutralization buffer: 2M Tris/Cl pH 8,0;

Chromatography was essentially done by following steps 1 to 7 described in table 11. In order to neutralize the low pH of the elution buffer "Fraction-tubes" were pre-loaded with 0.2 ml 2M Tris pH 8.0.

	STEP	BUFFER	Flow rate	Vol.	CV	Fractions
1.	column-wash	elution-buffer	2,0 ml/min	10 ml	5	waste
2.	equilibration	equi-buffer	2,0 ml/min	10 ml	5	waste
3.	sample-load	sample	1,0 ml/min	x ml	x	flow-through
4.	wash 1	equi-buffer	1,0 ml/min	20 ml	10	flow-through
5.	wash 2	wash-buffer	1,0 ml/min	10 ml	5	flow-through
6.	elution	elution-buffer	1,0 ml/min	15 ml	7,5	1,0 ml fractions-
7.	neutralization	wash-buffer	2,0 ml/min	10 ml	5	waste

Table 11

The final 198/A1 Fab preparation was dialyzed against 50mM imidazole, 100mM NaCl, pH7.4 and analyzed in a chromogenic FVIII assay as described above (Fig. 25). Compared to an intact antibody, the 198/A1 Fab fragment

has somewhat less activity; however, the Fab fragment still gives rise to FIX dependent FXa generation.

Fig. 25 demonstrates the chromogenic FVIII-like activity of the antibody 198/A1 Fab fragment in the presence of 2.3nM human FIXa. As a positive control we used the intact antibody 198/A1 as well as 7.5pM FVIII. Buffer control (IZ) instead of 198/A1 Fab fragment or FVIII was used as a negative control.

Example 16: FVIII-like activity exhibited by fusion proteins between scFv fragments of anti-FIX/FIXa antibodies and E. coli alkaline phosphatase.

The single chain Fv fragment (see example 10) of antibody 198/B1 (subclone AB2) was fused to the N-terminus of E. coli alkaline phosphatase employing the pDAP2 vector system (Kerschbaumer et al., 1996). Two identical clones were isolated and designated pDAP2-198AB2#1 and pDAP2-198AB2#100 (Fig. 26). The resulting fusion proteins were expressed in E. coli, purified by metal affinity chromatography (Kerschbaumer et al., 1997) and analysed in a standard chromogenic assay (Fig. 27).

Fig. 27 demonstrates the chromogenic FVIII-like activity of two antibody 198/B1 (subclone AB2) scFv fragment-alkaline phosphatase fusion proteins (198AB2#1 and 198AB2#100) in the presence of 2.3nM human FIXa. As a positive control we used 7.5pM FVIII.

Example 17: FVIII-like activity exhibited by a bivalent miniantibody.

In order to obtain a bivalent miniantibody, the scFv fragment of antibody 198/B1 (subclone AB2) was fused to a amphipatic helical structure employing the pZip1 vector system (Kerschbaumer et al. (*Analytical Biochemistry* 249, 219-227, 1997). Briefly, the gene of

the 198/B1 scFv fragment was isolated from the plasmid pDAP-198AB2#100 (example 16) by digestion with SfiI and NotI. The DNA fragment was gel purified and inserted in the SfiI/NotI digested vector pZip1. The resulting plasmid was sequenced and designated pZip-198AB2#102 (Fig.28). In parallel, we constructed a miniantibody version from an irrelevant monoclonal antibody termed #8860. In a first step, the single chain Fv fragment of antibody #8860 was assembled in the vector pDAP2. The cloning was done essentially as described in example 10. The construct was named pDAP2-8860scFv#11 (Fig. 29). Subcloning of the scFv fragment contained within pDAP2-8860scFv#11 into plasmid pZip1 (see above) yielded the miniantibody construct p8860-Zip#1.2 (Fig. 30). Since antibody #8860 does not react with FIX/FIXa (as judged by Western Blot and ELISA analysis) it represents an appropriate negative control. Subsequently, the miniantibody proteins were expressed in E. coli and purified from bacterial supernatants by binding to Protein L according to the following protocol:
For affinity chromatography we used ImmunoPure Immobilized PROTEIN L Plus (Pierce) in a PHARMACIA XK 16/20 Columns having a gel-volume of 4ml
Buffers employed were: 1) equilibration-buffer : 50mM L-Histidine pH 7.0, 1M NaCl, 0.1% (w/v) NaN_3 ; wash-buffer: 50mM L-histidine pH 7.0, 0.1% (w/v) NaN_3; elution-buffer: 100 mM glycine pH 2.5, 0.1% (w/v) NaN_3; and neutralization buffer: 2M Tris/Cl pH 8.0

Samples were prepared as follows: The bacterial culture supernatant was obtained by centrifugation of the bacterial expression culture (11,000 x g, 4°C, 10 minutes). 470 g of ammonium-sulphate was added to 1

liter of supernatant and the solution stirred on ice for 1 hour to precipitate the protein. The precipitate was pelleted at 14,000 x g for 35 minutes at 2°C and re-dissolved in 100 ml 20mM Tris pH 7.0. Subsequently the concentrate was dialyzed against 20mM Tris pH 7.0, L-histidine was added to a final concentration of 50mM and the pH was adjusted to 7.0. Finally, solid NaCl was added to give a final concentrations of 1M. Before loading on the column, a sample was first centrifuged at 16,000 x g for 15 min at room temperature and then filtered through a 0.45µm sterile filter.

Chromatography was essentially done by following steps 1 to 7 described in table 12. In order to neutralize the low pH of the elution buffer "Fraction-tubes" were pre-loaded with 0.2 ml 2M Tris pH 8.0.

	STEP	BUFFER	Flow rate	Vol.	CV	Fractions
1.	column-wash	elution-buffer	2.0 ml/min	20 ml	5	waste
2.	equilibration	equi-buffer	2.0 ml/min	20 ml	5	waste
3.	sample-load	sample	1.0 ml/min	x ml	x	flow-through
4.	wash 1	equi-buffer	1.0 ml/min	40 ml	10	flow-through
5.	wash 2	wash-buffer	1.0 ml/min	20 ml	5	flow-through
6.	elution	elution-buffer	1.0 ml/min	30 ml	7.5	1,0 ml fractions-
7.	neutralization	wash-buffer	2.0 ml/min	20 ml	5	waste

Table 12. The final 198/B1 (subclone AB2) miniantibody preparation (designated 198AB-Zip#102) and the negative control 8860-Zip#1.2 were dialyzed against 50mM imidazole, 100mM NaCl, pH7.4 and analyzed in a

chromogenic FVIII assay as described above (Fig. 31).

As can be seen in Fig. 31, the miniantibody construct 198AB-Zip#102 gives rise to substantial FXa generation (compare to FVIII) whereas the negative control miniantibody 8860-Zip#1.2 does not.
Fig. 31 demonstrates the chromogenic FVIII-like activity of the 198/B1 (subclone AB2) miniantibody 198AB-Zip#102 in the presence of 2.3nM human FIXa. As a positive control we used 4.8pM FVIII whereas an unrelated miniantibody (8860-Zip#1.2) and plain reaction buffer (IZ) served as negative controls.

Example 18: FVIII-like activity exhibited by anti-FIXa/FIX antibody scFv fragments

The single chain Fv fragment of antibody 198/B1 (subclone AB2) as well as the scFv fragment of antibody #8860 were expressed employing the pMycHis6 vector system. Vector pMycHis6 (Fig. 32 & 33) was constructed by cleaving vector pCOCK (Engelhardt et al., 1994, Biotechniques, 17:44-46) with NotI and EcoRI and insertion of the following oligonucleotides: mychis6-co: 5'ggccgcagaacaaaaactcatctcagaagaggatct gaatggggcggcacatcaccatcaccatcactaataag 3' (SEQ.ID.NO. 79) and mycchis-ic:5'aattcttattagtgatggtgatggtgatgtgccgccccattcagatcctct tctgagatgagttttgttctgc 3' (SEQ.ID.NO. 80)
Fig. 32 shows a schematic representation of the plasmid pMycHis6. The c-myc-tag sequence is used to detect the scFv fragment in an ELISA or a Western Blot analysis (Evan et al., Mol.Cell.Biol., 1985, 5(12), pp. 3610-6). The His6-tag sequence was included to facilitate the purification of scFv fragments by metal ion chromatography (Hochuli et al., 1988. Biotechnology,

6:1321-1325). The plasmid contains the lacZ gene promoter (PlacZ) the PelB-leader sequence (see legend Fig. 26) an E. coli origin of replication (colE1ori) and a M13 phage origin of replication (M13ori). To allow for specific selection, the plasmid also carries the gene for the enzyme ß-lactamase (AmpR) mediating resistance against the antibiotic ampicillin.

The gene of the 198/B1 (clone AB2)-scFv was rescued from plasmid pDAP2-198AB2#100 (example 16) by digestion with SfiI and NotI and inserted into SfiI/NotI cleaved pMycHis6. The resultant plasmid was designated pMycHis-198AB2#102. Fig. 34 shows the nucleotide and amino acid sequence of 198AB2 scFv (linked to the c-myc-tag and the His6- tag):the resulting ORF of the expression vector is named pMycHis6-198AB2#102. Vector pMycHis6 was constructed by cleaving vector pCOCK (Engelhardt O. et al, BioTechniques 17, 44-46, 1994) NotI - EcoRI and inserting the following annealed oligonucleotides:
(5'-GGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCTGAATGGG
GCGGCACATCACCATCACCATCACTAATAAG - 3' (SEQ.ID.No. 103)
and 5'- TTATTAGTGATGGTGATGGT
GATGTGCCGCCCCATTCAGATCCTCTTCTGAGATGAGTTTTTGTTCTGC-
3'(SEQ.ID.NO. 104)). The resultant vector, named pMycHis6, was cleaved SfiI - NotI and the gene of scFv 198AB2 was swapped into this vector from vector pDAP2-198AB2#100.

In analogy to the 198AB2 construct, the #8860 scFv fragment was cloned from a plasmid designated pDAP2-8860scFv clone 11. The pure scFv protein of #8860 was designated 8860-M/H#4c (plasmid p8860-M/H#4c, Fig. 35). The scFv proteins were expressed in E. coli and affinity purified from bacterial supernatants on Protein L columns (see example 17). The final MycHis-198AB2#102

and 8860-M/H#4c preparations were dialyzed against 50mM imidazole, 100mM NaCl, pH7.4 and analyzed in a chromogenic FVIII assay as described above (Fig. 36).

As can be seen in Fig. 36, the scFv construct MycHis-198AB2#102 gave rise to a substantial FXa generation whereas the negative controls 8860-M/H#4c and plain reaction buffer (IZ) did not.

Fig. 36 demonstrates the chromogenic FVIII-like activity of the 198/B1 (subclone AB2) scFv fragment (MycHis-198AB2#102) in the presence of 2.3nM human FIXa. As a positive control we used 4.8pM FVIII whereas a unrelated scFv (8860-M/H#4c) and plain reaction buffer (IZ) served as negative controls.

Claims:

1. An antibody or antibody derivative against factor IX/factor IXa which increases the procoagulant activity of FIXa.

2. An antibody or antibody derivative according to claim 1, wherein said antibody or antibody derivative increases the procoagulant activity of FIXa in the presence of FVIII inhibitors.

3. An antibody according to any one of claim 1 wherein said antibody is selected from the group consisting of IgG, IgM, IgA and IgE antibodies.

4. An antibody or antibody derivative according to claim 1, wherein said antibody or antibody derivative is selected from the group consisting of monoclonal antibodies, antibody fragments, chimeric antibodies, humanized antibodies, single chain antibodies, bispecific antibodies, diabodies, and di-, oligo- or multimers thereof.

5. An antibody derivative according to claim 1, wherein said antibody derivative comprises a complement determining region (CDR) peptide.

6. An antibody derivative according to claim 5, wherein said CDR peptide is a CDR3 peptide.

7. An antibody derivative according to claim 6, wherein said CDR3 peptide comprises an amino acid sequence selected from the group consisting of:

Tyr-Gly-Asn-Ser-Pro-Lys-Gly-Phe-Ala-Tyr;

Cys-X-X-Tyr-Gly-Asn-Ser-Pro-Lys-Gly-Phe-Ala-Tyr-X-X-Cys, wherein

X may be any desired amino acid;

Tyr-Gly-Asn-Ser-Pro-Lys-Gly-Phe-Ala-Tyr;

Asp-Gly-Gly-His-Gly-Tyr-Gly-Ser-Ser-Phe-Asp-Tyr; and

Phe-Arg-Asn-Arg-Gly-Met-Thr-Ala-Leu-Leu-Lys-Val-Ser-Ser-Cys-Asp.

8. An antibody or antibody derivative according to claim 1, wherein the variable region of said antibody or antibody derivative comprises amino acids 1 to 357 and/or amino acids 403 to 726 according to Fig. 14.

9. An antibody or antibody derivative according to claim 8, wherein said antibody or antibody derivative additionally comprises an artificial linker sequence.

10. An antibody or antibody derivative according to claim 1, wherein the variable region of said antibody or antibody derivative comprises amino acids 1 to 363 and/or amino acids 409 to 747 according to Fig. 15.

11. An antibody or antibody derivative according to claim 10, wherein said antibody or antibody derivative additionally comprises an artifical linker sequence.

12. An antibody or antibody derivative according to claim 1, ,wherein the variable region of said antibody

or antibody derivative comprises amino acids 1 to 366 and/or amino acids 412 to 747 according to Fig. 16.

13. An antibody or antibody derivative according to claim 12, wherein said antibody or antibody derivative additionally comprises an artificial linker sequence.

14. A hybridoma cell line expressing an antibody or antibody derivative against factor IX/factor IXa according to claim 1.

15. A hybridoma cell line according to claim 14, wherein said cell line is selected from the group consisting of #196/AF1, #196/AF2, #193/AD3, #193/K2-1, #198/AC1/1, #198/AM1, #198/A1, #198/B1, #198/AP1, 198/A1, 198/B1, 198/BB1, 198/A1, 198/B1, 198/BB1.

16. An antibody or antibody derivative according to claim 1, which is expressed by a hybridoma cell line according to claim 14.

17. A DNA molecule, wherein said DNA molecule encodes an antibody or an antibody derivative according to claim 1.

18. A pharmaceutical preparation comprising an antibody or antibody derivative according to claim 1and a pharmaceutically acceptable carrier.

19. A preparation according to claim 18, additionally comprising factor IXaα and/or factor IXaβ.

20. A method for treating patients afflicted with blood coagulation disorders comprising administering a pharmaceutically effective amount of the preparation of claim 18 to said patients.

21. The method of claim 20, wherein said blood coagulation disorders are selected from the group comprising hemophilia A and hemorrhagic diathesis.

22. The method of claim 21, additionally comprising the step of selecting hemophilia inhibitor patients.

23. A method of obtaining an antibody or antibody derivative which intereacts with factor IX/factor Ixa and increases the procoagulant activity of Factor IXa, comprising the steps of:

- immunizing an immunocompetent mouse with an antigen selected from the group consisting of FIX, FIXaα, FIXaβ or fragments thereof,
- isolating spleen cells of the immunized mouse,
- producing hybridoma clones,
- screening the hybridoma cell supernatants for an increase in the procoagulant activity of Factor Ixa, isolating and purifying the antibodies or antibody derivatives from hybridoma cell supernatants which exhibit an increase in the procoagulant activity of factor IXa.

24. Use of an antibody or antibody derivative according to claim 1 for increasing the amidolytic activity of factor IXa.

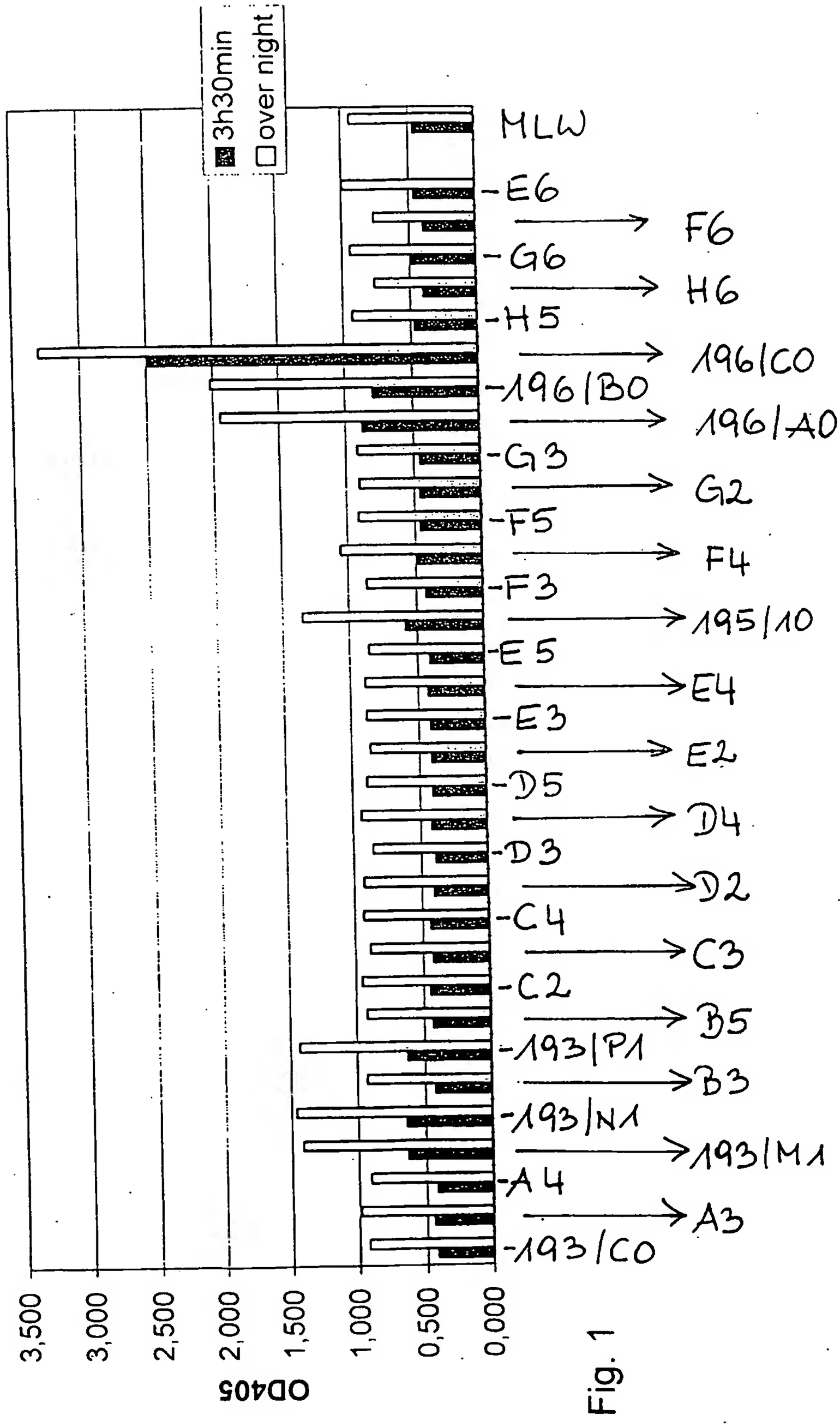
3h30min
over night
MLW
-E6
F6
-G6
H6
-H5
196/C0
-196/B0
196/A0
-G3
G2
-F5
F4
-F3
195/10
-E5
E4
-E3
E2
-D5
D4
-D3
D2
-C4
C3
-C2
B5
-193/P1
B3
-193/N1
193/M1
-A4
A3
-193/C0
3,500
3,000
2,500
2,000
1,500
1,000
0,500
0,000
OD405
clone #

Fig. 1

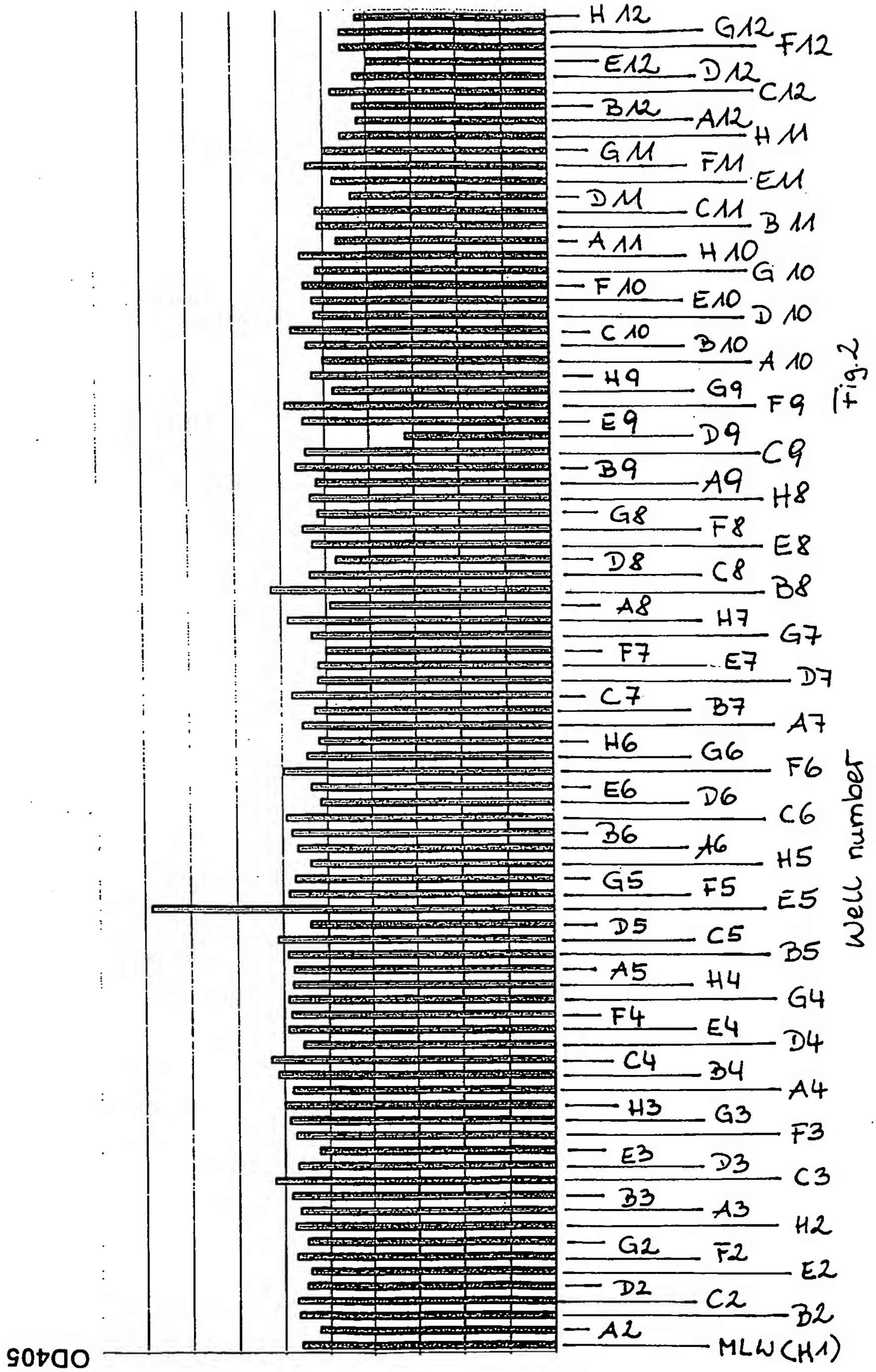
H12
G12
F12
E12
D12
C12
B12
A12
H11
G11
F11
E11
D11
C11
B11
A11
H10
G10
F10
E10
D10
C10
B10
A10
H9
G9
F9
E9
D9
C9
B9
A9
H8
G8
F8
E8
D8
C8
B8
A8
H7
G7
F7
E7
D7
C7
B7
A7
H6
G6
F6
E6
D6
C6
B6
A6
H5
G5
F5
E5
D5
C5
B5
A5
H4
G4
F4
E4
D4
C4
B4
A4
H3
G3
F3
E3
D3
C3
B3
A3
H2
G2
F2
E2
D2
C2
B2
A2
MLW(H1)
OD405
Well number

Fig. 2

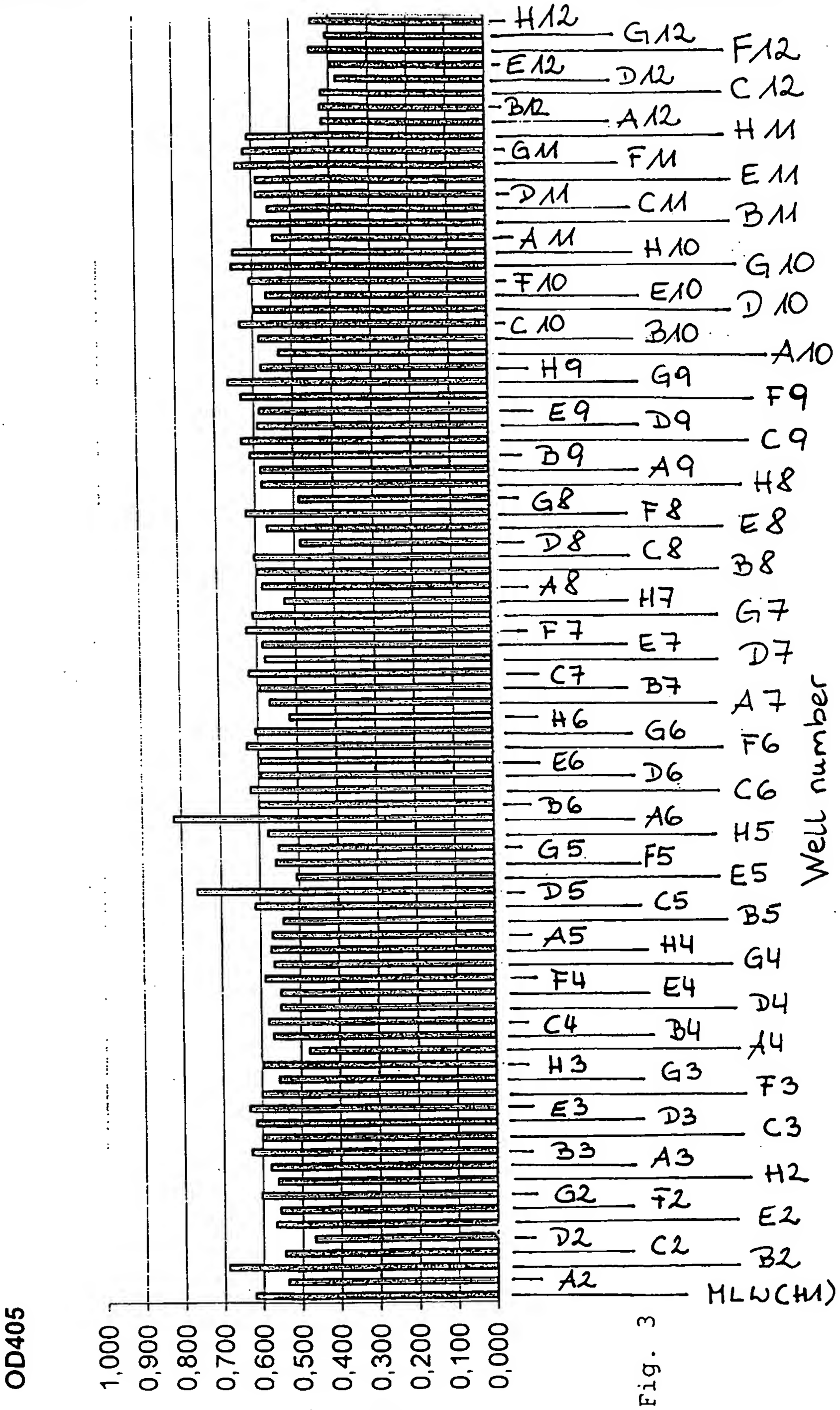
H12
G12
F12
E12
D12
C12
B12
A12
H11
G11
F11
E11
D11
C11
B11
A11
H10
G10
F10
E10
D10
C10
B10
A10
H9
G9
F9
E9
D9
C9
B9
A9
H8
G8
F8
E8
D8
C8
B8
A8
H7
G7
F7
E7
D7
C7
B7
A7
H6
G6
F6
E6
D6
C6
D6
A6
H5
G5
F5
E5
D5
C5
B5
A5
H4
G4
F4
E4
D4
C4
B4
A4
H3
G3
F3
E3
D3
C3
B3
A3
H2
G2
F2
E2
D2
C2
B2
A2
MLW(H1)
Well number
OD405
1,000
0,900
0,800
0,700
0,600
0,500
0,400
0,300
0,200
0,100
0,000

Fig. 3

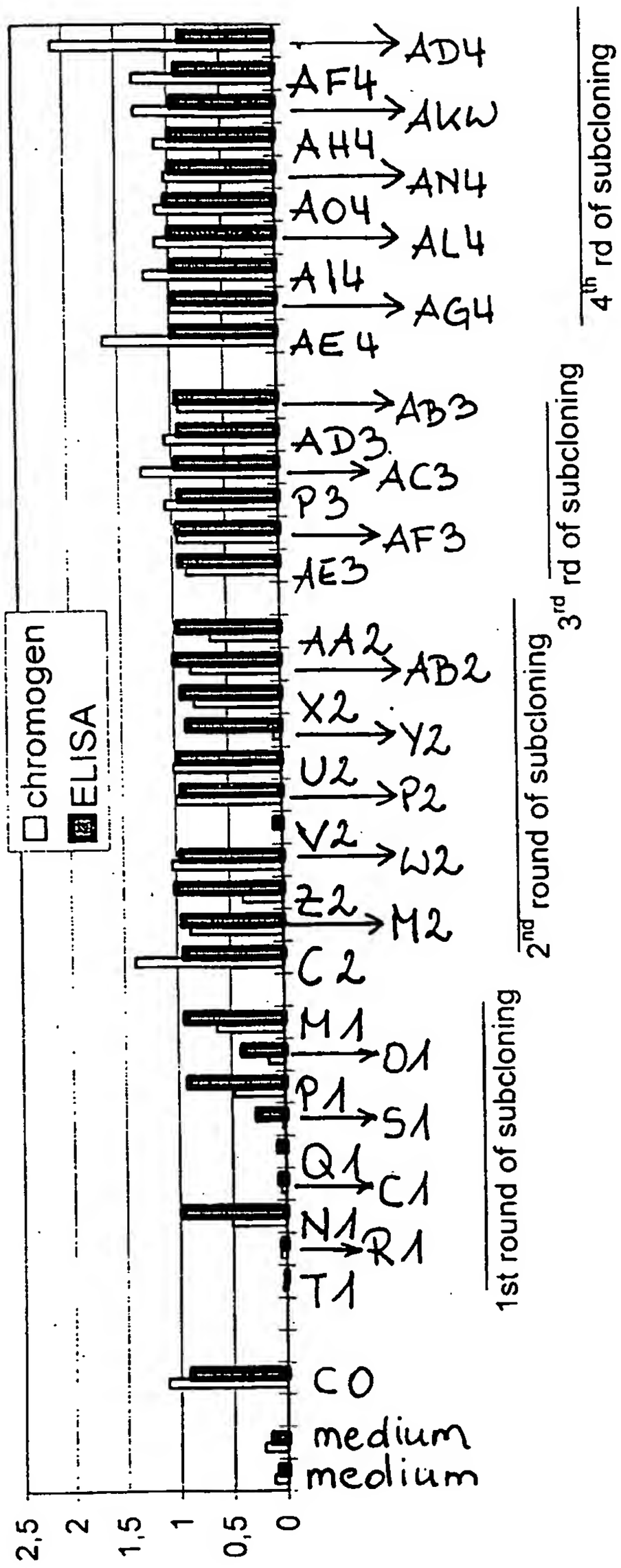
AD4
AF4
AKW
AH4
AN4
AO4
AL4
AI4
AG4
AE4
AB3
AD3
AC3
P3
AF3
AE3
AA2
AB2
X2
Y2
U2
P2
V2
W2
Z2
M2
C2
M1
O1
P1
S1
Q1
C1
N1
R1
T1
CO
medium
medium
chromogen
ELISA
2,5
2
1,5
1
0,5
0
4th rd of subcloning
3rd rd of subcloning
2nd round of subcloning
1st round of subcloning

Fig. 4

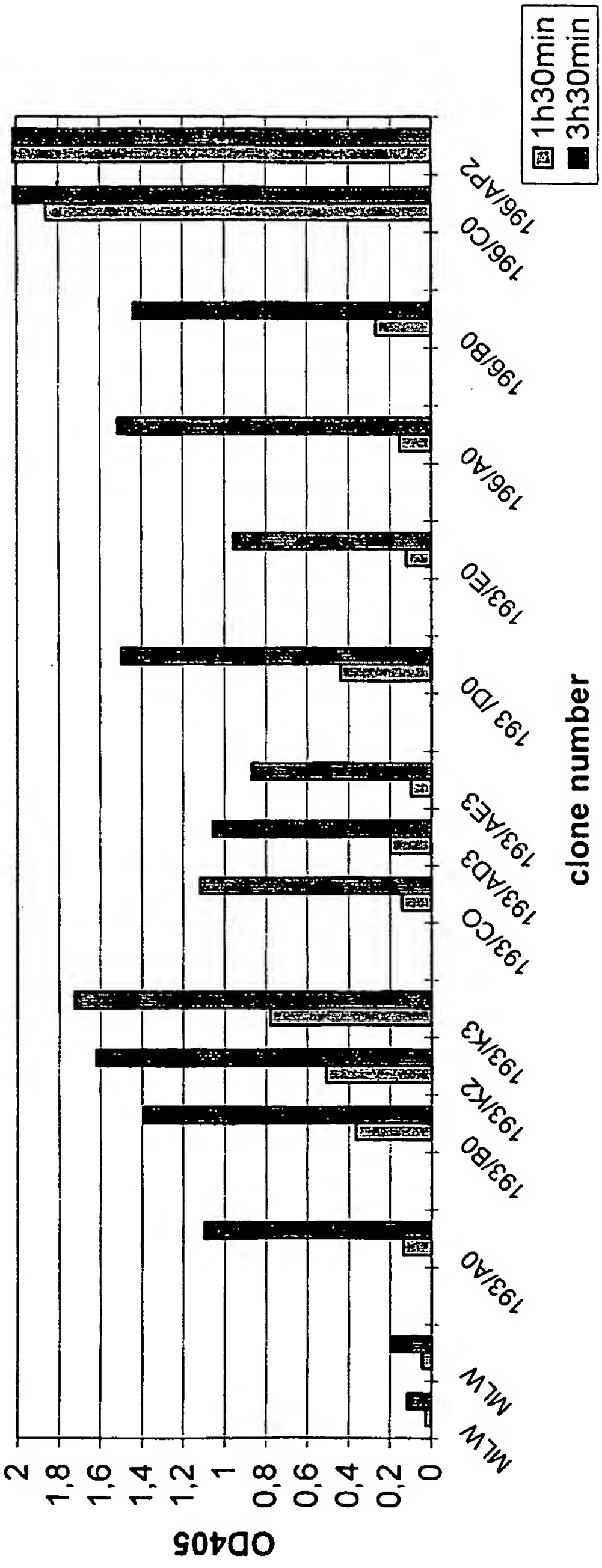
1h30min
3h30min
clone number
OD405
0
0,2
0,4
0,6
0,8
1
1,2
1,4
1,6
1,8
2
MLW
MLW
MLW
193/A0
193/B0
193/K2
193/K3
193/C0
193/AD3
193/AE3
193 /D0
193/E0
196/A0
196/B0
196/C0
196/AP2

Fig. 5

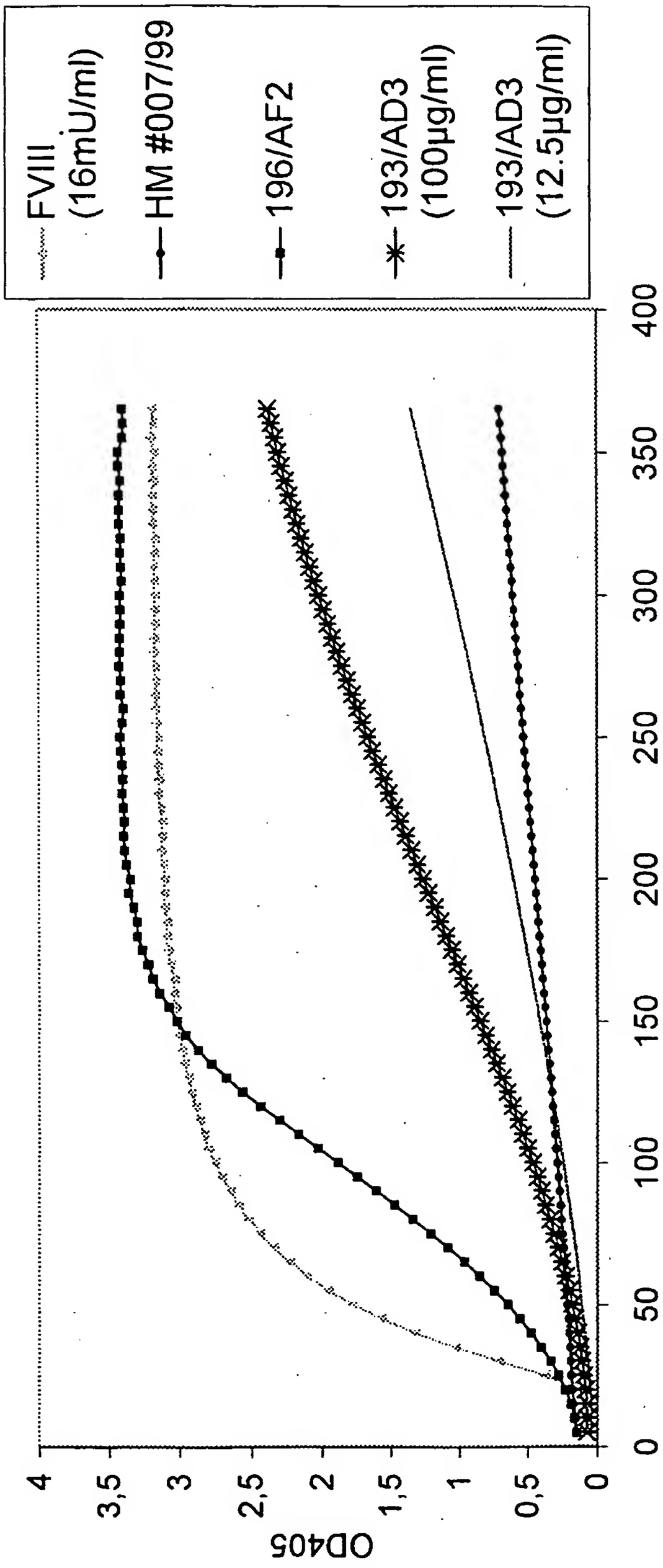
FVIII (16mU/ml)
HM #007/99
196/AF2
193/AD3 (100µg/ml)
193/AD3 (12.5µg/ml)
OD405
0
0,5
1
1,5
2
2,5
3
3,5
4
0
50
100
150
200
250
300
350
400
Time (min)

Fig. 6A

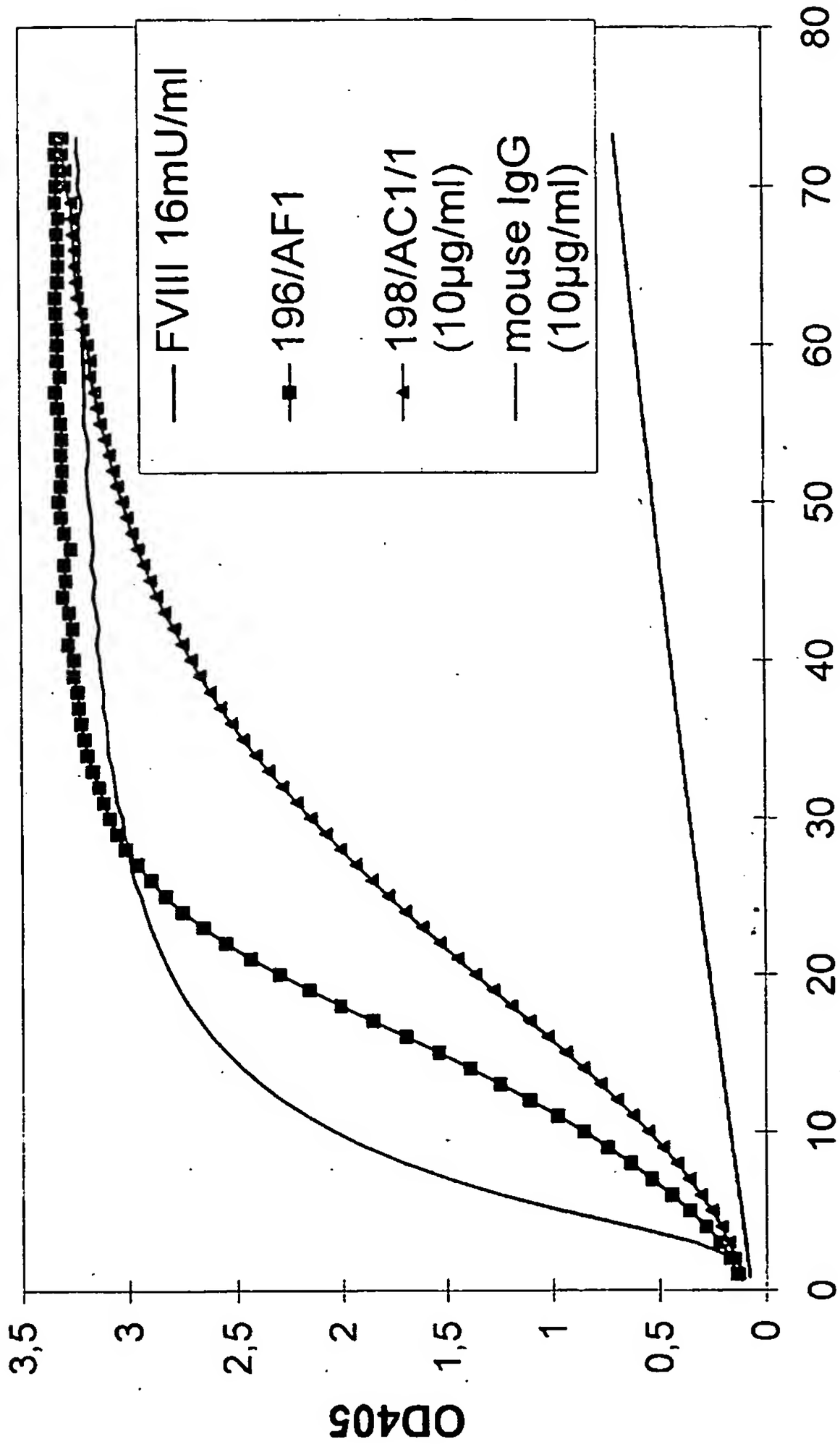
OD405
3,5
3
2,5
2
1,5
1
0,5
0
0
10
20
30
40
50
60
70
80
Scan Number
FVIII 16mU/ml
196/AF1
198/AC1/1 (10µg/ml)
mouse IgG (10µg/ml)

Fig. 6B

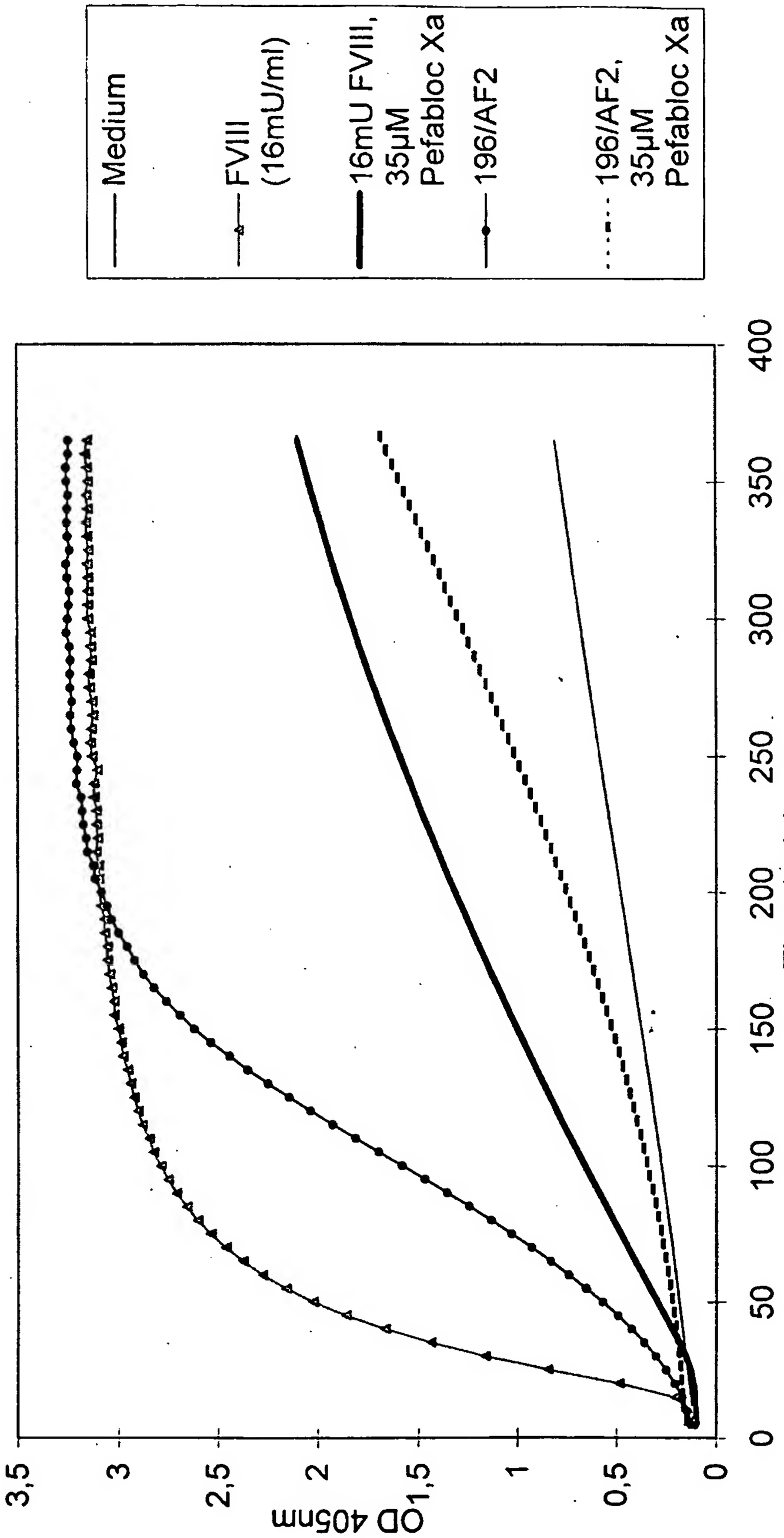
Medium
FVIII (16mU/ml)
16mU FVIII, 35µM Pefabloc Xa
196/AF2
196/AF2, 35µM Pefabloc Xa
400
350
300
250
200
150
100
50
0
Time (min)
OD 405nm
3,5
3
2,5
2
1,5
1
0,5
0

Fig. 7A

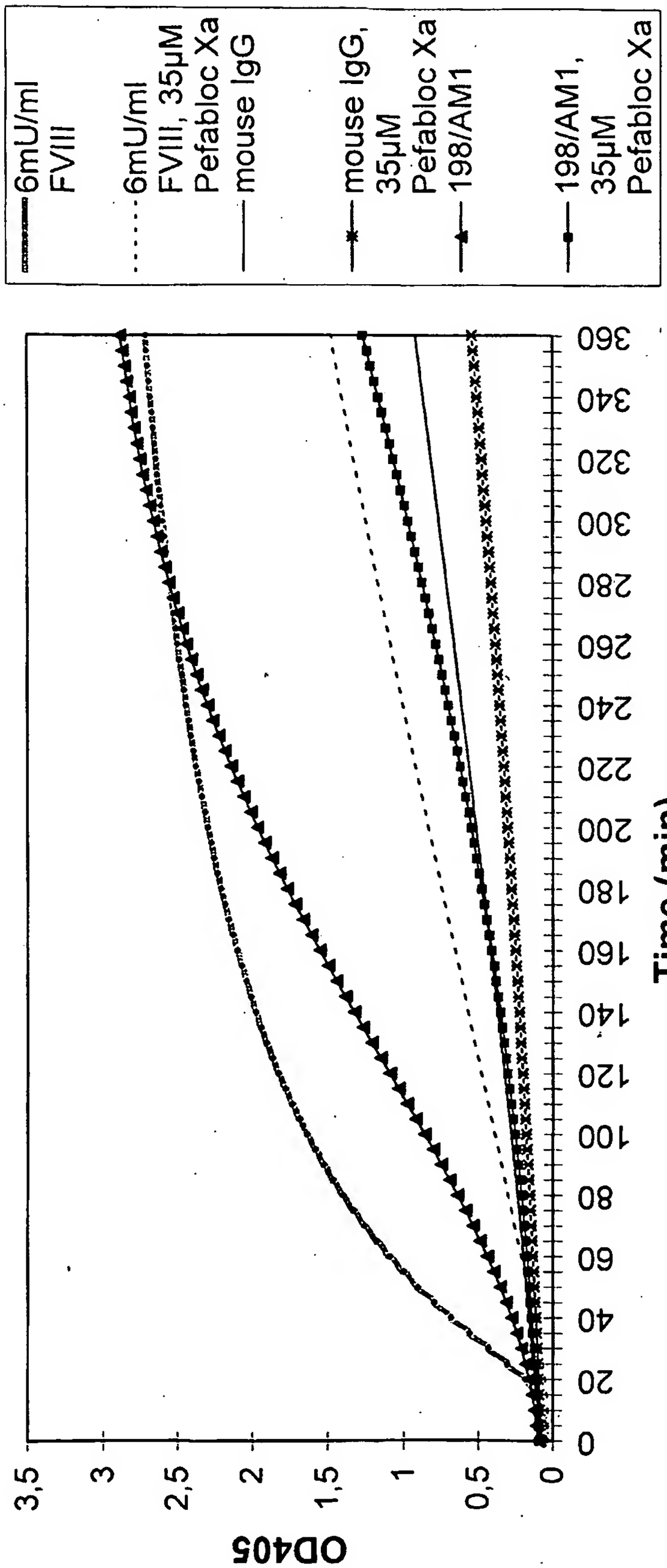
6mU/ml FVIII
6mU/ml FVIII, 35µM Pefabloc Xa
mouse IgG
mouse IgG, 35µM Pefabloc Xa
198/AM1
198/AM1, 35µM Pefabloc Xa
OD405
Time (min)
3,5
3
2,5
2
1,5
1
0,5
0
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360

Fig. 7B

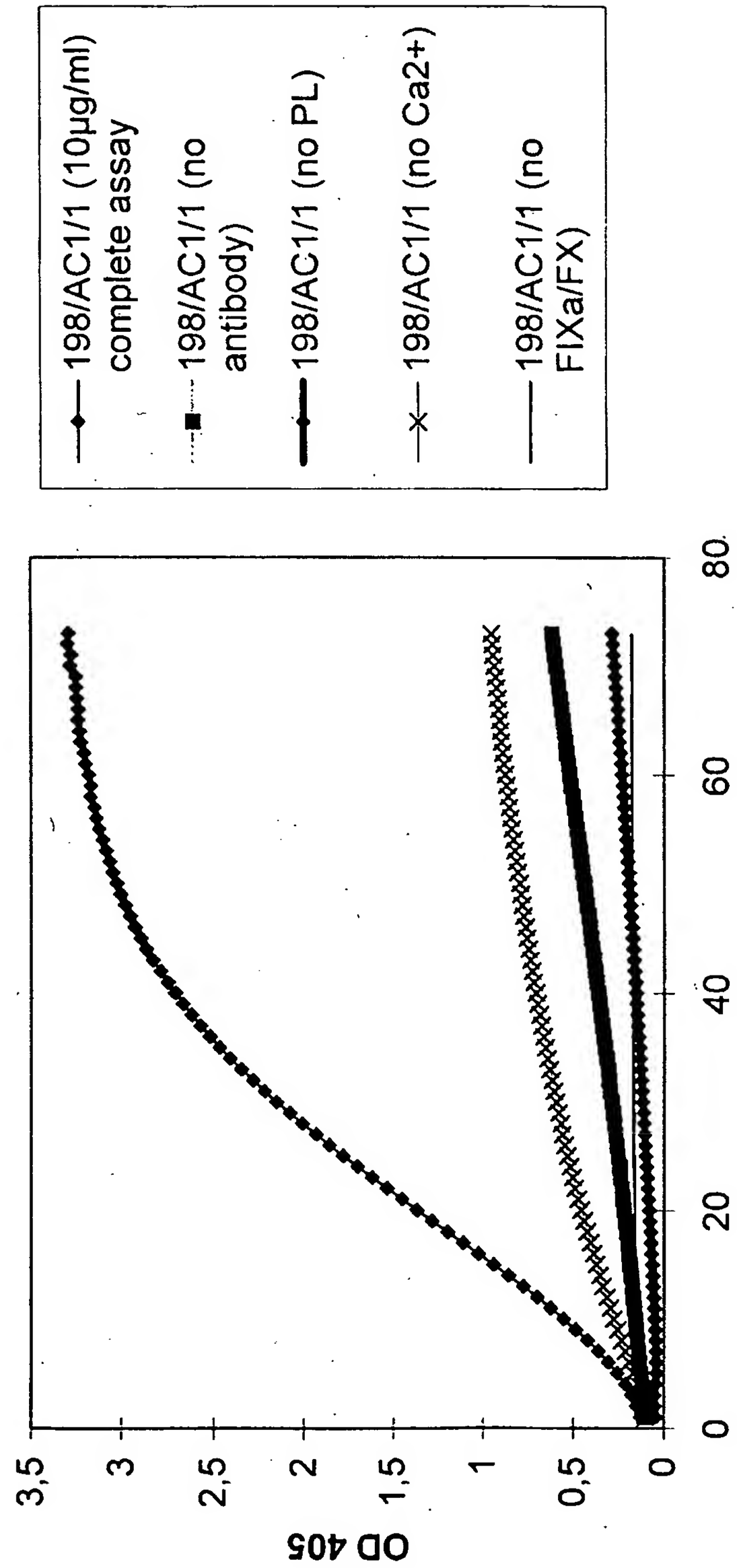
198/AC1/1 (10µg/ml) complete assay
198/AC1/1 (no antibody)
198/AC1/1 (no PL)
198/AC1/1 (no Ca2+)
198/AC1/1 (no FIXa/FX)
OD 405
Scan number
0
0,5
1
1,5
2
2,5
3
3,5
0
20
40
60
80

Fig. 8A

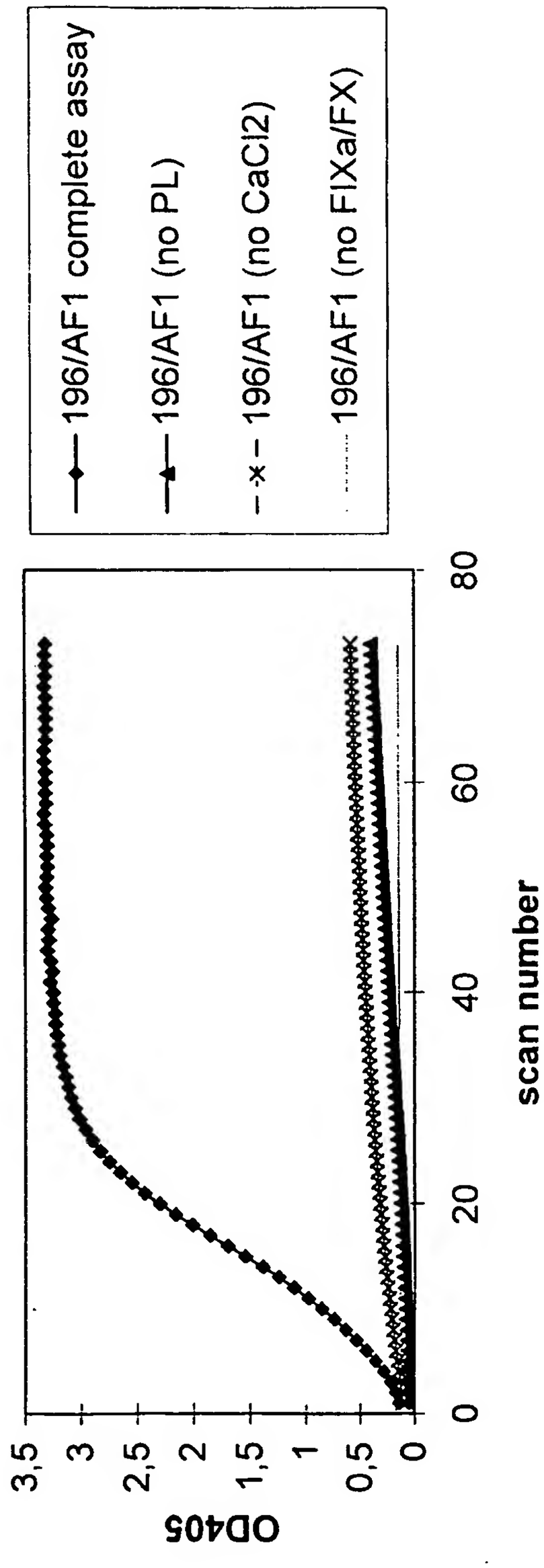
196/AF1 complete assay
196/AF1 (no PL)
196/AF1 (no CaCl2)
196/AF1 (no FIXa/FX)
OD405
3,5
3
2,5
2
1,5
1
0,5
0
0
20
40
60
80
scan number

Fig. 8B

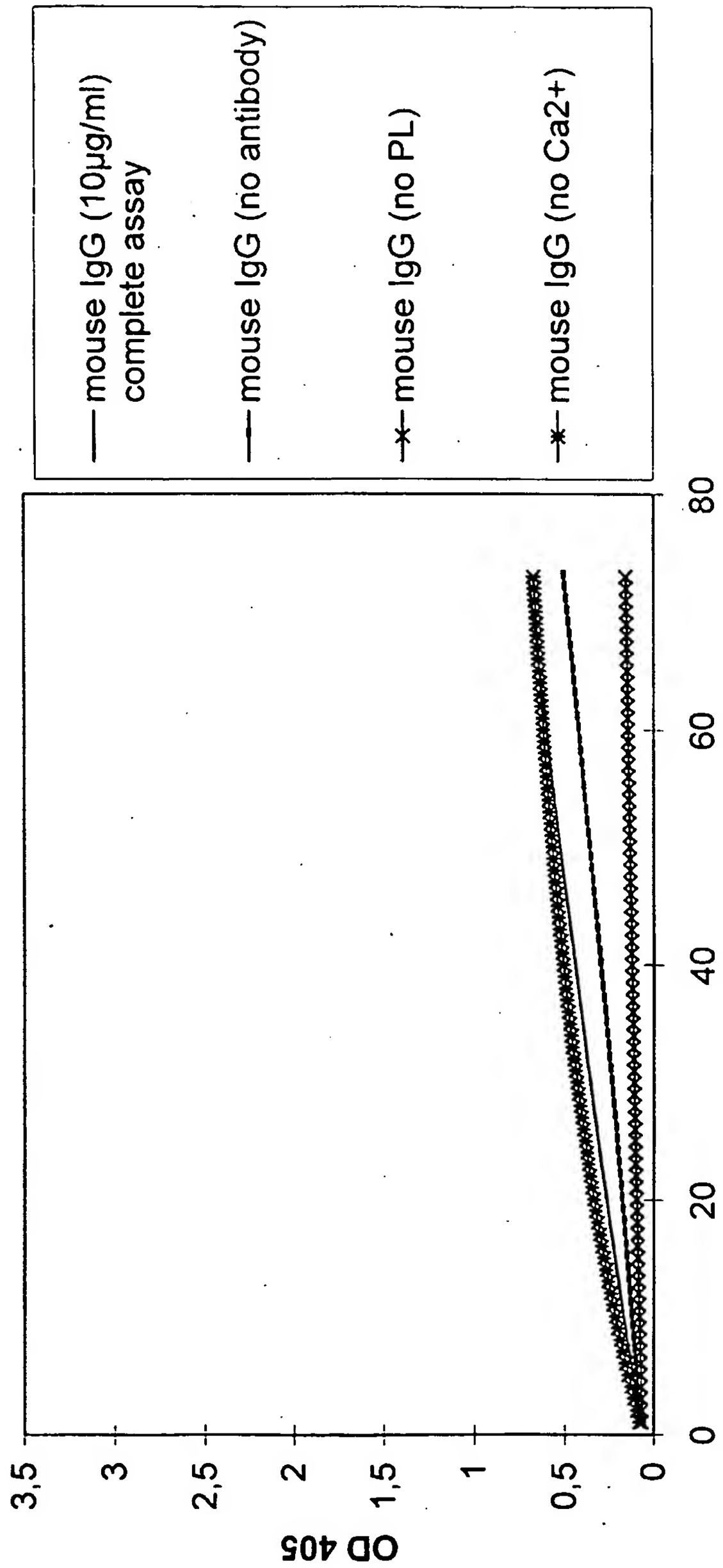
mouse IgG (10µg/ml) complete assay
mouse IgG (no antibody)
mouse IgG (no PL)
mouse IgG (no Ca2+)
OD 405
3,5
3
2,5
2
1,5
1
0,5
0
0
20
40
60
80
Scan number

Fig. 8C

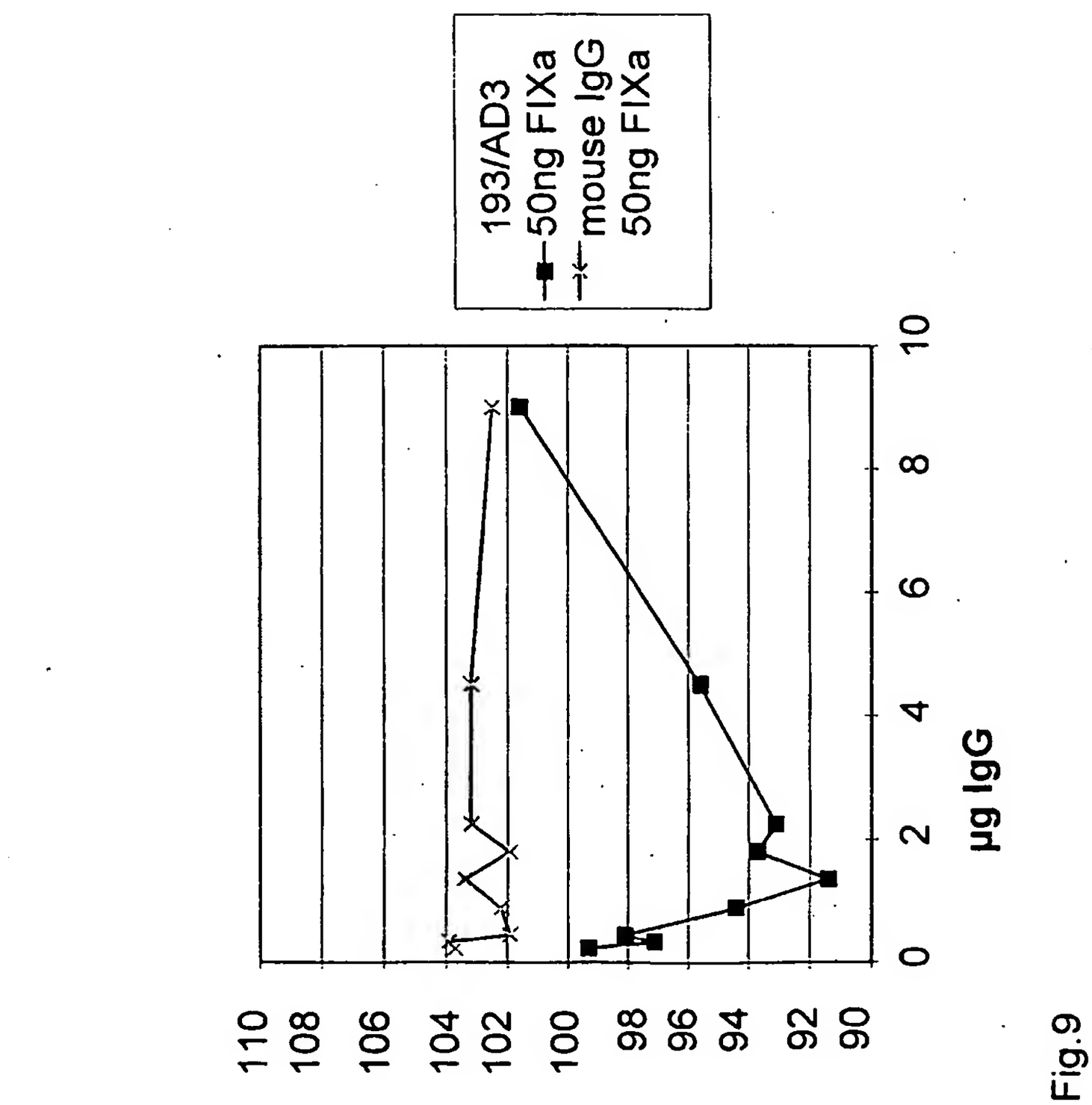
193/AD3
50ng FIXa
mouse IgG
50ng FIXa
µg IgG
0
2
4
6
8
10
90
92
94
96
98
100
102
104
106
108
110

Fig.9

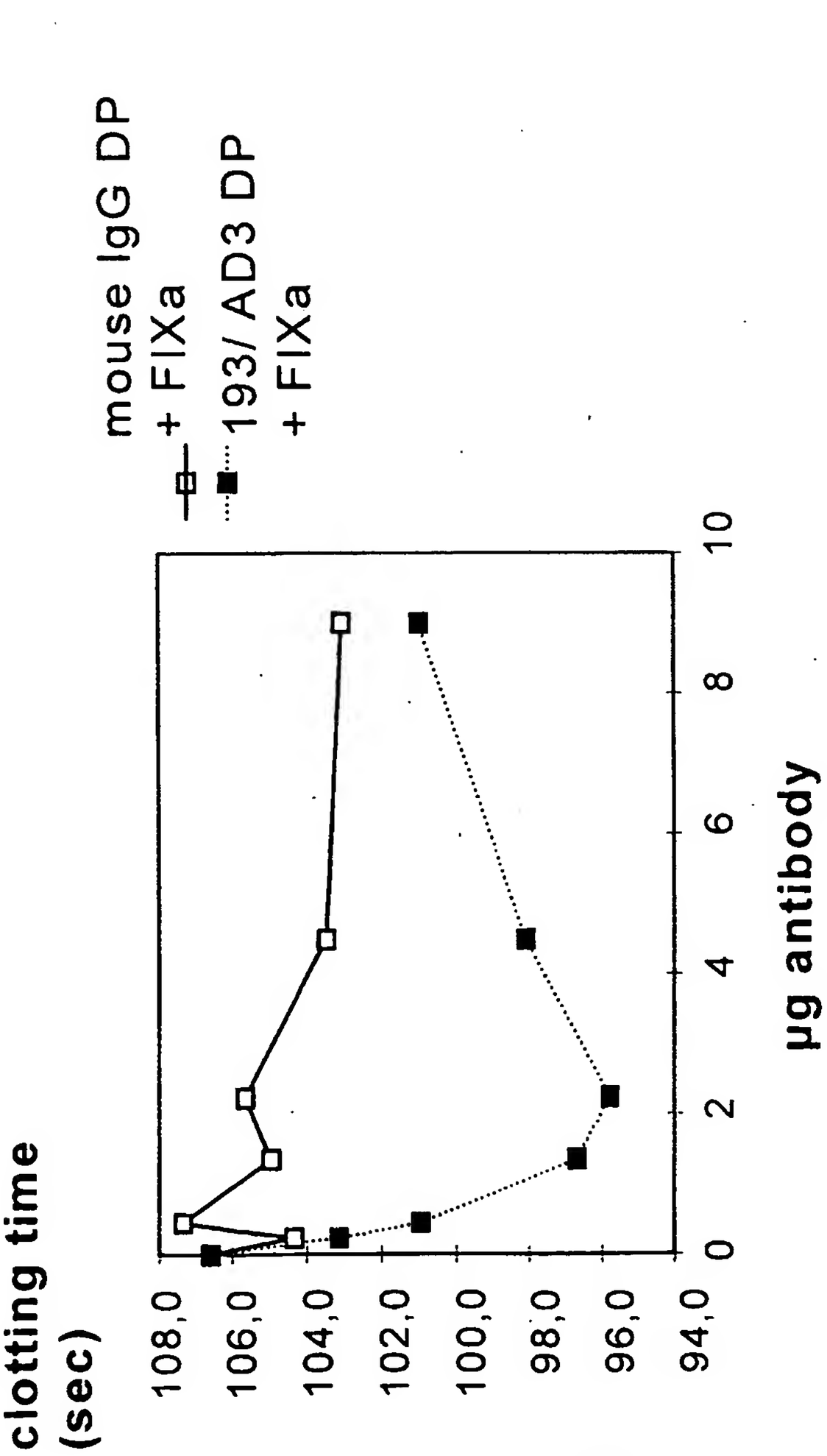
mouse IgG DP
+ FIXa
193/ AD3 DP
+ FIXa
clotting time
(sec)
108,0
106,0
104,0
102,0
100,0
98,0
96,0
94,0
0
2
4
6
8
10
µg antibody

Fig. 10A

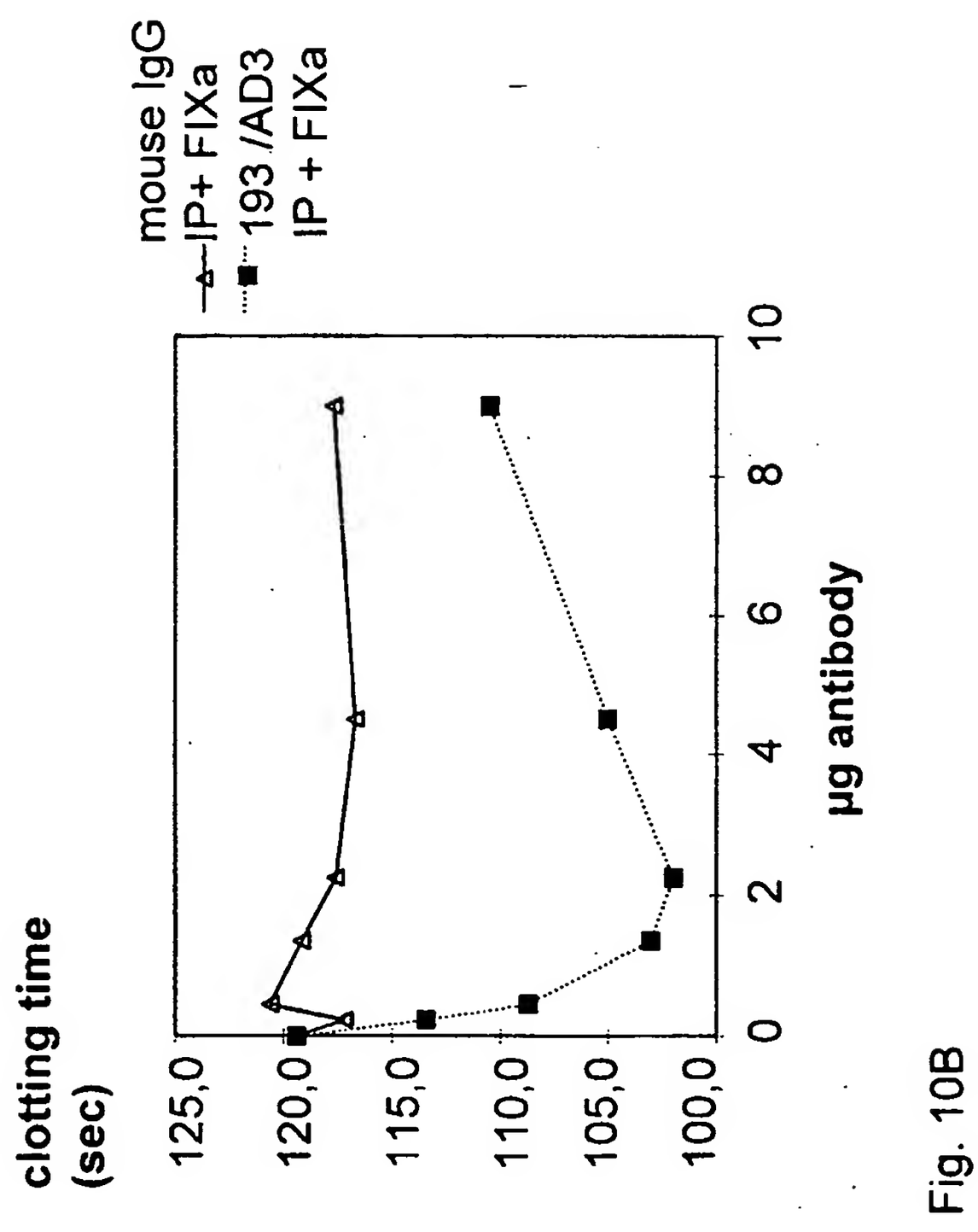
mouse IgG
IP+ FIXa
193 /AD3
IP + FIXa
clotting time
(sec)
125,0
120,0
115,0
110,0
105,0
100,0
0
2
4
6
8
10
µg antibody

Fig. 10B

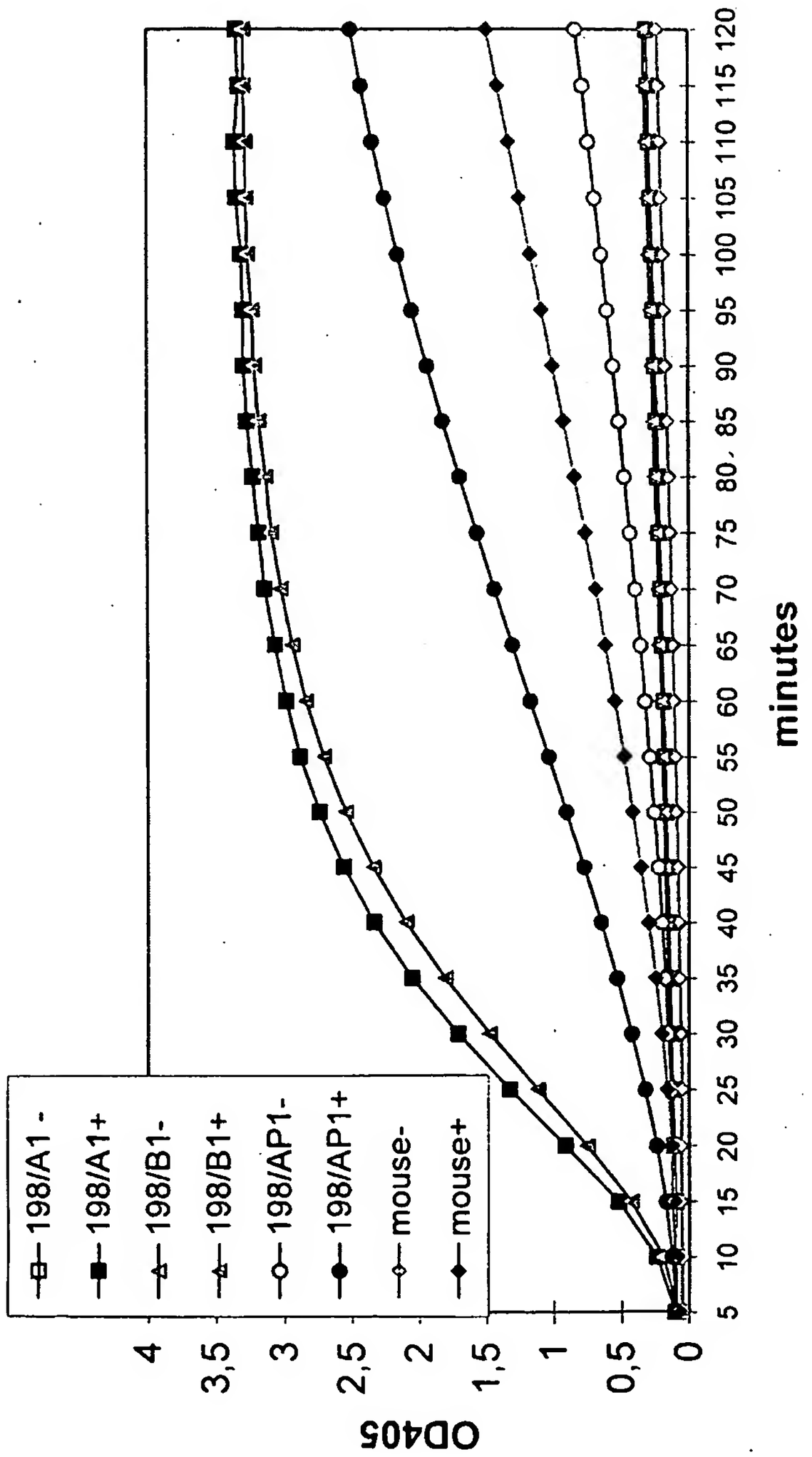
OD405
minutes
198/A1 -
198/A1+
198/B1-
198/B1+
198/AP1-
198/AP1+
mouse-
mouse+
0
0,5
1
1,5
2
2,5
3
3,5
4
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120

Fig.11

Mouse V_H back primers (containing SfiI-site):

VH1BACK-SfiI 5′ C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC SAG GTS MAR CTG CAG SAG TCW GG 3′ (SEQ.ID.NO. 50)

VH1BACKSfi 5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTG CAG CTT CAG GAG TCA GG 3′ (SEQ.ID.NO. 51)

VH2BACKSfi 5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAT GTG CAG CTT CAG GAG TCR GG 3′ (SEQ.ID.NO. 52)

VH3BACKSfi 5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG AAG SAG TCA GG 3′ (SEQ.ID.NO. 53)

VH4/6BACKSfi 5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTY CAG CTG CAR CAR TCT GG 3′ (SEQ.ID.NO. 54)

VH5/9BACKSfi 5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTY CAR CTG CAG CAG YCT GG 3′ (SEQ.ID.NO. 55)

VH7BACKSfi 5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAR GTG AAG CTG GTG GAR TCT GG 3′ (SEQ.ID.NO. 56)

VH8BACKSfi 5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTT CAG CTT CAG CAG TCT GG 3′ (SEQ.ID.NO. 57)

VH10BACKSfi 5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAA GTG CAG CTG KTG GAG WCT GG 3′ (SEQ.ID.NO. 58)

VH11BACKSfi 5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG ATC CAG TTG CTG CAG TCT GG 3′ (SEQ.ID.NO. 59)

FIG. 12-1

Mouse J_H forward primers (containing ½ linker-sequence and AscI-site):

VH1FOR2LiAsc 5′ ACC GCC AGA GGC GCG CCC ACC TGA ACC GCC TCC ACC TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC 3′(SEQ.ID.NO. 60)

JH1FORLiAsc 5′ ACC GCC AGA GGC GCG CCC ACC TGA ACC GCC TCC ACC TGA GGA GAC GGT GAC CGT GGT CCC 3′ (SEQ.ID.NO. 61)

JH2FORLiAsc 5′ ACC GCC AGA GGC GCG CCC ACC TGA ACC GCC TCC ACC TGA GGA GAC TGT GAG AGT GGT GCC 3′(SEQ.ID.NO. 62)

JH3FORLiAsc 5′ ACC GCC AGA GGC GCG CCC ACC TGA ACC GCC TCC ACC TGC AGA GAC AGT GAC CAG AGT CCC 3′ (SEQ.ID.NO. 63)

JH4FORLiAsc 5′ ACC GCC AGA GGC GCG CCC ACC TGA ACC GCC TCC ACC TGA GGA GAC GGT GAC TGA GGT TCC 3′(SEQ.ID.NO. 64)

IUPAC-Code: M=A/C, W=A/T, R=A/G, Y=C/T, S=C/G, K=G/T, H=A/C/T, D=A/G/T, V=A/C/G, B=T/C/G.

Fig. 12 -2

Primers for cloning mouse V_K genes

Mouse V_K back primers (containing AscI-site and ½ linker-sequence):

VK2BACK-LiAscI	5′ GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG GAC ATT GAG CTC ACC CAG TCT CCA 3′ (SEQ.ID.NO. 65)
VK1BACKLi Asc	5′ GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG GAC ATT GTG ATG WCA CAG TCT CC 3′ (SEQ.ID.NO. 66)
VK2BACKLi Asc	5′ GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG GAT GTT KTG ATG ACC CAA ACT CC 3′ (SEQ.ID.NO. 67)
VK3BACKLi Asc	5′ GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG GAT ATT GTG ATR ACB CAG GCW GC 3′ (SEQ.ID.NO. 68)
VK4BACKLi Asc	5′ GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG GAC ATT GTG CTG ACM CAR TCT CC 3′ (SEQ.ID.NO. 69)
VK5BACKLi Asc	5′ GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG SAA AWT GTK CTC ACC CAG TCT CC 3′ (SEQ.ID.NO. 70)
VK6BACKLi Asc	5′ GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG GAY ATY VWG ATG ACM CAG WCT CC 3′ (SEQ.ID.NO. 71)
VK7BACKLi Asc	5′ GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG CAA ATT GTT CTC ACC CAG TCT CC 3′ (SEQ.ID.NO. 72)
VK8BACKLi Asc	5′ GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG TCA TTA TTG CAG GTG CTT GTG GG 3′ (SEQ.ID.NO. 73)

Fig. 13-1

Mouse J_K forward primers (containing NotI-site):

JK1NOT10	5′ GAG TCA TTC TGC GGC CGC CCG TTT GAT TTC CAG CTT GGT GCC 3′
	(SEQ.ID.NO. 74)
JK2NOT10	5′ GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAG CTT GGT CCC 3′
	(SEQ.ID.NO. 75)
JK3NOT10	5′ GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAG TCT GGT CCC 3′
	(SEQ.ID.NO.76)
JK4NOT10	5′ GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAA CTT TGT CCC 3′
	(SEQ.ID.NO. 77)
JK5NOT10	5′ GAG TCA TTC TGC GGC CGC CCG TTT CAG CTC CAG CTT GGT CCC 3′
	(SEQ.ID.NO. 78)

IUPAC-Code: K=G/T, M=A/C, W=A/T, R=A/G, Y=C/T, S=C/G, H=A/C/T, D=A/G/T, V=A/C/G, B=T/C/G.

Fig. 13-2

VH

```
+1    E   V   K   L   V   E   S   G   P   E   L   K   K   P   G
1   GAG GTG AAG CTG GTG GAG TCT GGA CCT GAG CTG AAG AAG CCT GGA

+1    E   T   V   K   I   S   C   K   A   S   G   Y   I   F   T
46  GAG ACA GTC AAG ATC TCC TGC AAG GCT TCT GGG TAT ATC TTC ACA

+1    N   Y   G   M   N   W   V   K   Q   A   P   G   K   G   L
91  AAC TAT GGA ATG AAC TGG GTG AAG CAG GCT CCA GGA AAG GGT TTA

+1      K   W   M   G   W   I   N   T   Y   T   G   E   P   T   Y
136   AAG TGG ATG GGC TGG ATA AAC ACC TAC ACT GGA GAG CCA ACA TAT

+1      A   D   D   F   K   G   R   F   A   F   S   L   E   T   S
181   GCT GAT GAC TTC AAG GGA CGG TTT GCC TTC TCT TTG GAA ACC TCT

+1      A   S   T   A   Y   L   Q   I   N   N   L   K   N   E   D
226   GCC AGC ACT GCC TAT TTG CAG ATC AAC AAC CTC AAA AAT GAG GAC

+1      T   A   T   Y   F   C   A   L   Y   G   N   S   P   K   G
271   ACG GCT ACA TAT TTC TGT GCA TTA TAT GGT AAC TCC CCT AAG GGG

                                                    linker
+1      F   A   Y   W   G   Q   G   T   L   V   T   V   S   A   G
316   TTT GCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA GGT

                                                    VL
+1      G   G   G   S   G   G   R   A   S   G   G   G   G   S   D
361   GGA GGC GGT TCA GGT GGG CGC GCC TCT GGC GGT GGC GGA TCG GAT

+1      I   Q   M   T   Q   S   P   K   F   L   L   V   S   A   G
406   ATT CAG ATG ACA CAG TCT CCC AAA TTC CTG CTT GTA TCA GCA GGA
```

Fig. 14-1

```
+1    D   R   V   T   I   T   C   K   A   S   Q   S   V   S   N
451   GAC AGG GTT ACC ATA ACC TGC AAG GCC AGT CAG AGT GTG AGT AAT

+1    D   V   A   W   Y   Q   Q   K   P   G   Q   S   P   K   L
496   GAT GTA GCT TGG TAC CAA CAG AAG CCG GGG CAG TCT CCT AAA CTA

+1    L   M   Y   Y   A   S   N   R   Y   T   G   V   P   D   R
541   CTG ATG TAC TAT GCA TCC AAT CGC TAC ACT GGA GTC CCT GAT CGC

+1    F   T   G   S   G   Y   G   T   D   F   T   F   T   I   S
586   TTC ACT GGC AGT GGA TAT GGG ACG GAT TTC ACT TTC ACC ATC AGC

+1    T   V   Q   A   E   D   L   A   V   Y   F   C   Q   Q   D
631   ACT GTG CAG GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG CAG GAT

+1    Y   G   S   P   P   T   F   G   G   G   T   K   L   E   I
676   TAT GGC TCT CCT CCC ACG TTC GGA GGG GGC ACC AAG CTG GAA ATT

+1    K   R
721   AAA CGG
```

Fig. 14 -2

VH

+1	E	V	Q	L	V	E	S	G	G	G	L	V	K	P	G
1	GAA	GTG	CAG	CTG	GTG	GAG	TCT	GGG	GGA	GGC	CTA	GTG	AAG	CCT	GGA
+1	G	S	L	K	L	S	C	A	A	S	G	F	T	F	S
46	GGG	TCC	CTG	AAA	CTC	TCC	TGT	GCA	GCC	TCT	GGA	TTC	ACT	TTC	AGT
+1	T	Y	T	M	S	W	V	R	Q	T	P	E	K	R	L
91	ACC	TAT	ACC	ATG	TCT	TGG	GTT	CGC	CAG	ACT	CCG	GAG	AAG	AGG	CTG
+1	E	W	V	A	T	I	S	S	G	G	S	Y	T	Y	Y
136	GAG	TGG	GTC	GCA	ACC	ATT	AGT	AGT	GGT	GGT	AGT	TAC	ACC	TAC	TAT
+1	P	D	S	V	R	G	R	F	T	I	S	R	D	N	A
181	CCA	GAC	AGT	GTG	AGG	GGC	CGA	TTC	ACC	ATC	TCC	AGA	GAC	AAT	GCC
+1	K	N	T	L	Y	L	Q	M	S	S	L	K	S	E	D
226	AAG	AAC	ACC	CTG	TAC	CTG	CAA	ATG	AGC	AGT	CTG	AAG	TCT	GAG	GAC
+1	T	A	M	Y	Y	C	T	R	D	G	G	H	G	Y	G
271	ACA	GCC	ATG	TAT	TAC	TGT	ACA	AGA	GAT	GGG	GGA	CAC	GGG	TAC	GGT
+1	S	S	F	D	Y	W	G	Q	G	T	T	L	T	V	S
316	AGT	AGC	TTT	GAC	TAC	TGG	GGC	CAA	GGC	ACC	ACT	CTC	ACA	GTC	TCC

linker

+1	S	*G*	*G*	*G*	*G*	*S*	*G*	*G*	*R*	*A*	*S*	*G*	*G*	*G*	*G*
361	TCA	*GGT*	*GGA*	*GGC*	*GGT*	*TCA*	*GGT*	*GGG*	*CGC*	*GCC*	*TCT*	*GGC*	*GGT*	*GGC*	*GGA*

VL

+1	*S*	Q	I	V	L	T	Q	S	P	L	S	L	P	V	S
406	*TCG*	CAA	ATT	GTG	CTC	ACC	CAG	TCT	CCA	CTC	TCC	CTG	CCT	GTC	AGT

Fig. 15-1

```
+1    L   G   D   Q   A   S   I   S   C   R   S   S   Q   S   I
451   CTT GGA GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC ATT

+1    V   H   S   N   G   N   T   Y   L   E   W   Y   L   Q   K
496   GTA CAT AGT AAT GGA AAC ACC TAT TTA GAA TGG TAC CTG CAG AAA

+1    P   G   Q   S   P   K   L   L   I   Y   K   V   S   N   R
541   CCA GGC CAG TCT CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA

+1    F   S   G   V   P   D   K   F   S   G   S   G   S   G   T
586   TTT TCT GGG GTC CCA GAC AAA TTC AGT GGC AGT GGA TCA GGG ACA

+1    D   F   T   L   K   I   S   R   V   E   A   E   D   L   G
631   GAT TTC ACA CTC AAG ATC AGC AGA GTG GAG GCT GAG GAT CTG GGA

+1    V   Y   Y   C   F   Q   G   S   H   V   P   W   T   F   G
676   GTT TAT TAC TGC TTT CAA GGT TCA CAT GTT CCG TGG ACG TTC GGT

1     G   G   T   K   L   E   I   K   R
721   GGA GGC ACC AAG CTG GAA ATC AAA CGG
```

Fig. 15-2

```
+1  E   V   Q   L   Q   E   S   G   G   G   L   V   K   P   G
  1 GAG GTG CAG CTT CAG GAG TCA GGG GGA GGC TTA GTG AAG CCT GGA

 +1  G   S   L   K   L   S   C   A   A   S   G   F   T   F   S
 46  GGG TCC CTG AAA CTC TCC TGT GCA GCC TCT GGA TTC ACT TTC AGT

 +1  S   Y   T   M   S   W   V   R   Q   T   P   E   K   R   L
 91  AGC TAT ACC ATG TCT TGG GTT CGC CAG ACT CCG GAG AAG AGG CTG

 +1  E   W   V   A   T   I   S   S   G   G   S   S   T   Y   Y
136  GAG TGG GTC GCA ACC ATT AGT AGT GGT GGT AGT TCC ACC TAC TAT

 +1  P   D   S   V   K   G   R   F   T   I   S   R   D   N   A
181  CCA GAC AGT GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT GCC

 +1  K   N   T   L   Y   L   Q   M   S   S   L   R   S   E   D
226  AAG AAC ACC CTG TAC CTG CAA ATG AGC AGT CTG AGG TCT GAG GAC

 +1  T   A   M   Y   Y   C   T   R   E   G   G   G   F   T   V
271  ACA GCC ATG TAT TAC TGT ACA AGA GAG GGG GGT GGT TTC ACC GTC

 +1  N   W   Y   F   D   V   W   G   A   G   T   L   V   T   V
316  AAC TGG TAC TTC GAT GTC TGG GGC GCA GGG ACT CTG GTC ACT GTC

              linker
 +1  S   A   G   G   G   G   S   G   G   R   A   S   G   G   G
361  TCT GCA GGT GGA GGC GGT TCA GGT GGG CGC GCC TCT GGC GGT GGC

              VL
 +1  G   S   E   N   V   L   T   Q   S   P   A   S   L   A   V
406  GGA TCG GAA AAT GTG CTC ACC CAG TCT CCA GCT TCT TTG GCT GTG
```

Fig. 16-1

```
+1  S   L   G   Q   R   A   T   I   S   C   R   A   S   E   S
451 TCT CTA GGG CAG AGG GCC ACC ATA TCC TGC AGA GCC AGT GAA AGT

 +1  V   D   S   Y   G   Y   N   F   M   H   W   Y   Q   Q   I
496 GTT GAT AGT TAT GGC TAT AAT TTT ATG CAC TGG TAT CAG CAG ATA

 +1  P   G   Q   P   P   K   L   L   I   Y   R   A   S   N   L
541 CCA GGA CAG CCA CCC AAA CTC CTC ATC TAT CGT GCA TCC AAC CTA

 +1  E   S   G   I   P   A   R   F   S   G   S   G   S   R   T
586 GAG TCT GGG ATC CCT GCC AGG TTC AGT GGC AGT GGG TCT AGG ACA

 +1  D   F   T   L   T   I   N   P   V   E   A   D   D   V   A
631 GAC TTC ACC CTC ACC ATT AAT CCT GTG GAG GCT GAT GAT GTT GCA

 +1  T   Y   Y   C   Q   Q   S   N   E   D   P   L   T   F   G
676 ACC TAT TAC TGT CAG CAA AGT AAT GAG GAT CCG CTC ACG TTC GGT

 +1  T   G   T   R   L   E   I   K   R
721 ACT GGG ACC AGA CTG GAA ATA AAA CGG
```

Fig.16 -2

VH

```
+1   E   V   Q   L   Q   E   S   G   G   G   L   V   K   P   G   G   S   L   K   L
1   GAG GTG CAG CTT CAG GAG TCA GGG GGA GGC TTA GTG AAG CCT GGA GGG TCC CTG AAA CTC
    CTC CAC GTC GAA GTC CTC AGT CCC CCT CCG AAT CAC TTC GGA CCT CCC AGG GAC TTT GAG

+1   S   C   A   A   S   G   F   I   F   S   S   Y   T   M   S   W   V   R   Q   T
61  TCC TGT GCA GCC TCT GGA TTC ATT TTT AGT AGT TAT ACC ATG TCT TGG GTT CGC CAG ACT
    AGG ACA CGT CGG AGA CCT AAG TAA AAA TCA TCA ATA TGG TAC AGA ACC CAA GCG GTC TGA

+1   P   E   K   R   L   E   W   V   A   T   I   S   S   G   G   S   S   T   Y   Y
121 CCG GAG AAG AGG CTG GAG TGG GTC GCA ACC ATT AGT AGT GGT GGT AGT TCC ACC TAC TAT
    GGC CTC TTC TCC GAC CTC ACC CAG CGT TGG TAA TCA TCA CCA CCA TCA AGG TGG ATG ATA

+1   P   D   S   V   K   G   R   F   T   I   S   R   D   N   A   K   N   T   L   Y
181 CCA GAC AGT GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC CTG TAC
    GGT CTG TCA CAC TTC CCG GCT AAG TGG TAG AGG TCT CTG TTA CGG TTC TTG TGG GAC ATG

+1   L   Q   M   S   S   L   K   S   E   D   T   A   M   Y   H   C   T   R   E   G
241 CTG CAA ATG AGC AGT CTG AAG TCT GAG GAC ACA GCC ATG TAT CAC TGT ACA AGA GAG GGG
    GAC GTT TAC TCG TCA GAC TTC AGA CTC CTG TGT CGG TAC ATA GTG ACA TGT TCT CTC CCC

+1   G   G   Y   Y   V   N   W   Y   F   D   V   W   G   A   G   T   T   L   T   V
301 GGT GGT TAT TAC GTC AAC TGG TAC TTC GAT GTC TGG GGC GCA GGC ACC ACT CTC ACA GTC
    CCA CCA ATA ATG CAG TTG ACC ATG AAG CTA CAG ACC CCG CGT CCG TGG TGA GAG TGT CAG
```

linker *VL*

```
+1   S   S   G   G   G   G   S   G   G   R   A   S   G   G   G   G   S   D   I   E
361 TCC TCA GGT GGA GGC GGT TCA GGT GGG CGC GCC TCT GGC GGT GGC GGA TCG GAC ATT GAG
    AGG AGT CCA CCT CCG CCA AGT CCA CCC GCG CGG AGA CCG CCA CCG CCT AGC CTG TAA CTC

+1   L   T   Q   S   P   A   S   L   A   V   S   L   G   Q   R   A   T   I   S   C
421 CTC ACN CAG TCT CCA GCT TCT TTG GCT GTG TCT CTA GGG CAG AGG GCC ACC ATA TCC TGC
    GAG TGN GTC AGA GGT CGA AGA AAC CGA CAC AGA GAT CCC GTC TCC CGG TGG TAT AGG ACG
```

Fig. 17-1

```
  +1  R   A   S   E   S   V   D   S   Y   G   K   S   F   M   H   W   Y   Q   Q   K
481  AGA GCC AGT GAA AGT GTT GAT AGT TAT GGC AAG AGT TTT ATG CAC TGG TAC CAG CAG AAA
     TCT CGG TCA CTT TCA CAA CTA TCA ATA CCG TTC TCA AAA TAC GTG ACC ATG GTC GTC TTT

  +1  P   G   Q   P   P   K   L   L   I   Y   R   A   S   N   L   E   S   G   I   P
541  CCA GGG CAG CCA CCC AAA CTC CTC ATC TAT CGT GCA TCC AAC CTA GAA TCT GGG ATC CCT
     GGT CCC GTC GGT GGG TTT GAG GAG TAG ATA GCA CGT AGG TTG GAT CTT AGA CCC TAG GGA

  +1  A   R   F   S   G   S   G   S   R   T   D   F   T   L   T   I   N   P   V   E
601  GCC AGG TTC AGT GGC AGT GGG TCT AGG ACA GAC TTC ACC CTC ACC ATT AAT CCT GTG GAG
     CGG TCC AAG TCA CCG TCA CCC AGA TCC TGT CTG AAG TGG GAG TGG TAA TTA GGA CAC CTC

  +1  A   D   D   V   A   T   Y   Y   C   Q   Q   S   N   E   D   P   L   T   F   G
661  GCT GAT GAT GTT GCN ACC TAT TAC TGT CAG CAA AGT AAT GAG GAT CCC CTC ACG TTC GGT
     CGA CTA CTA CAA CGN TGG ATA ATG ACA GTC GTT TCA TTA CTC CTA GGG GAG TGC AAG CCA

  +1  A   G   T   R   L   E   I   K   R
721  GCT GGG ACC AGA CTG GAA ATA AAA CGG
     CGA CCC TGG TCT GAC CTT TAT TTT GCC
```

Fig. 17-2

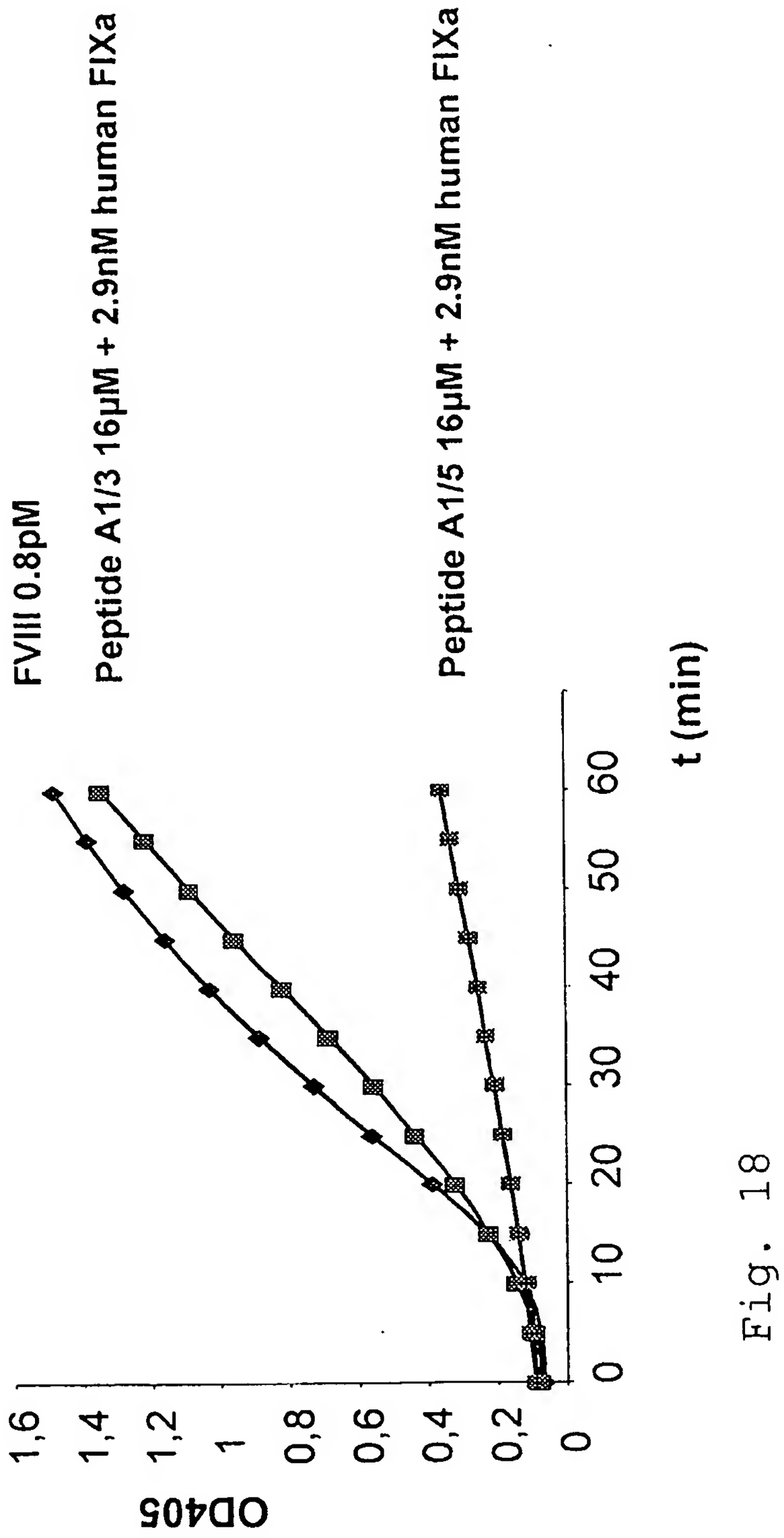
FVIII 0.8pM
Peptide A1/3 1.6µM + 2.9nM human FIXa
Peptide A1/5 1.6µM + 2.9nM human FIXa
OD405
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0
10
20
30
40
50
60
t (min)

Fig. 18

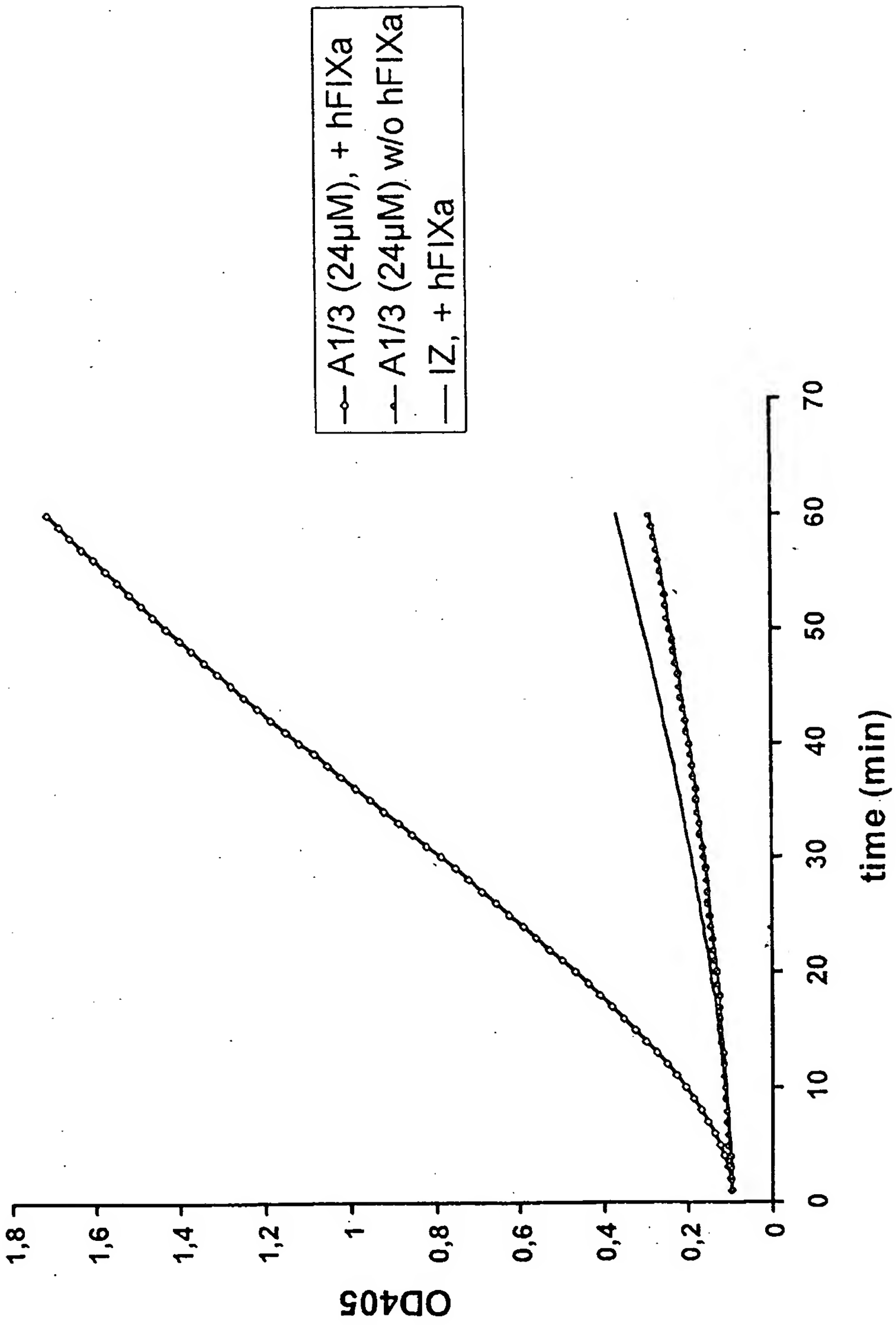
A1/3 (24µM), + hFIXa
A1/3 (24µM) w/o hFIXa
IZ, + hFIXa
OD405
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0
10
20
30
40
50
60
70
time (min)

Fig. 19

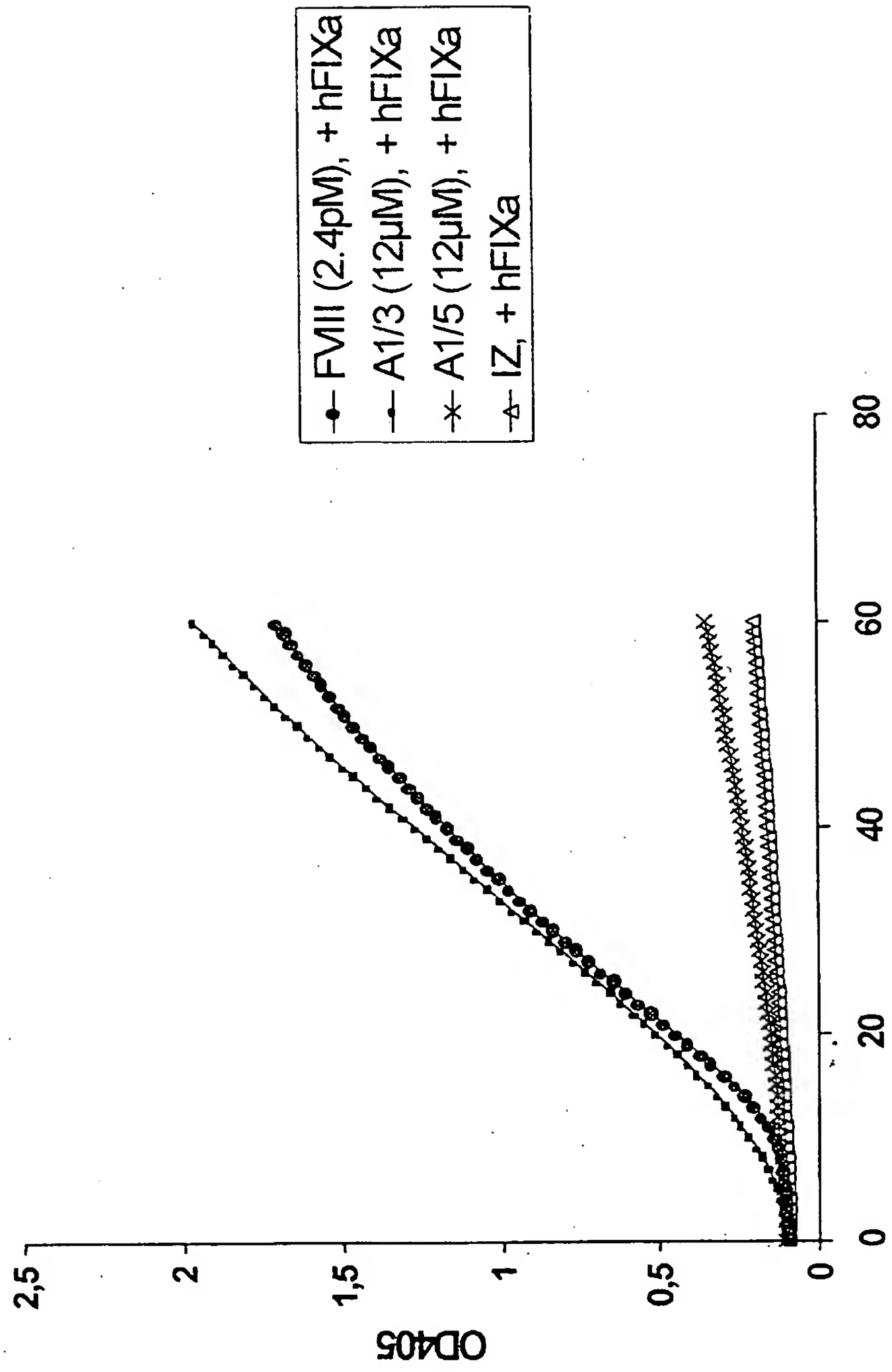
FVIII (2.4pM), + hFIXa
A1/3 (12µM), + hFIXa
A1/5 (12µM), + hFIXa
IZ, + hFIXa
OD405
2,5
2
1,5
1
0,5
0
0
20
40
60
80
time (min)

Fig. 20

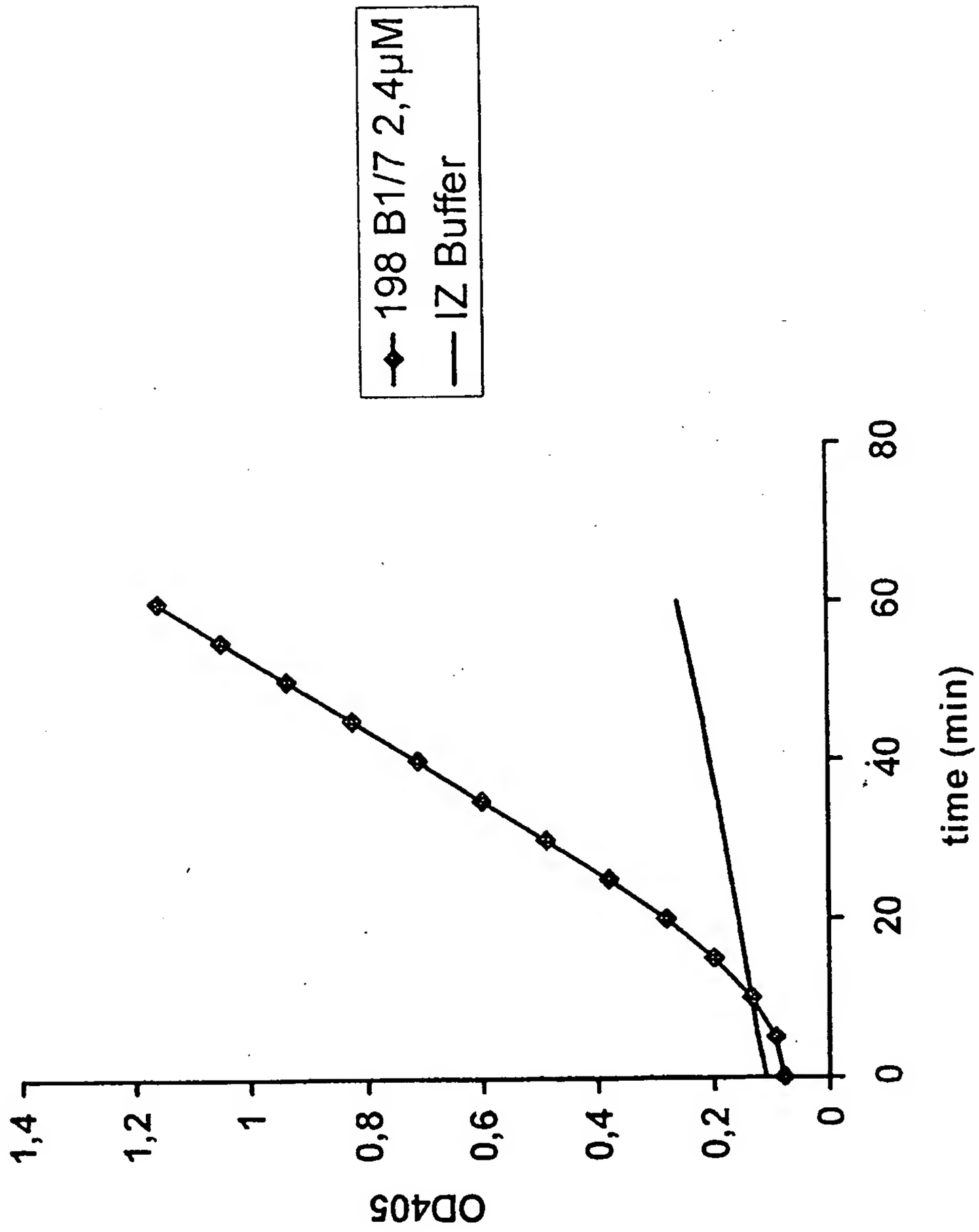
198 B1/7 2,4µM
IZ Buffer
OD405
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0
20
40
60
80
time (min)

Fig. 21

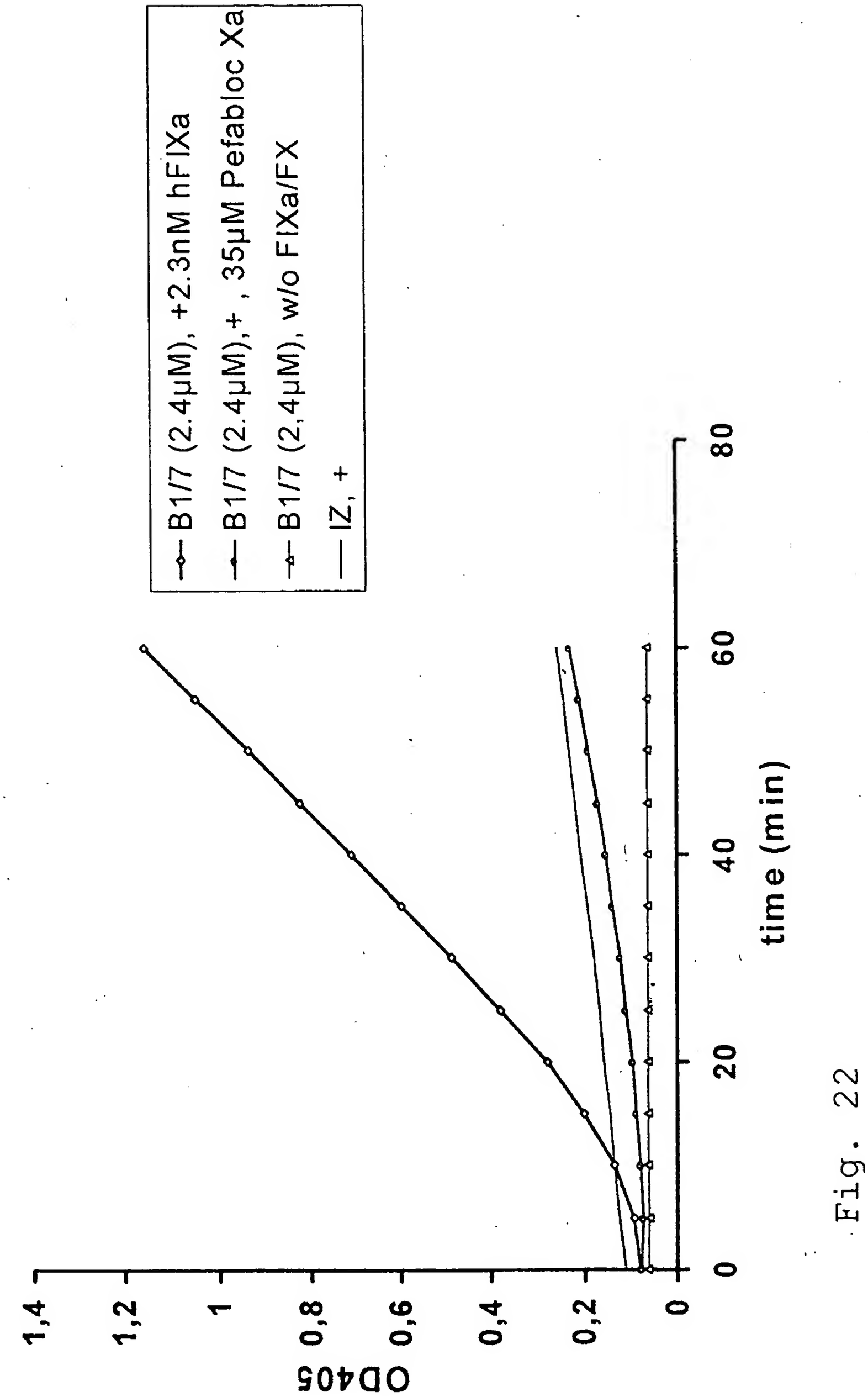
B1/7 (2.4µM), +2.3nM hFIXa
B1/7 (2.4µM),+ , 35µM Pefabloc Xa
B1/7 (2,4µM), w/o FIXa/FX
IZ, +
OD405
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0
20
40
60
80
time (min)

Fig. 22

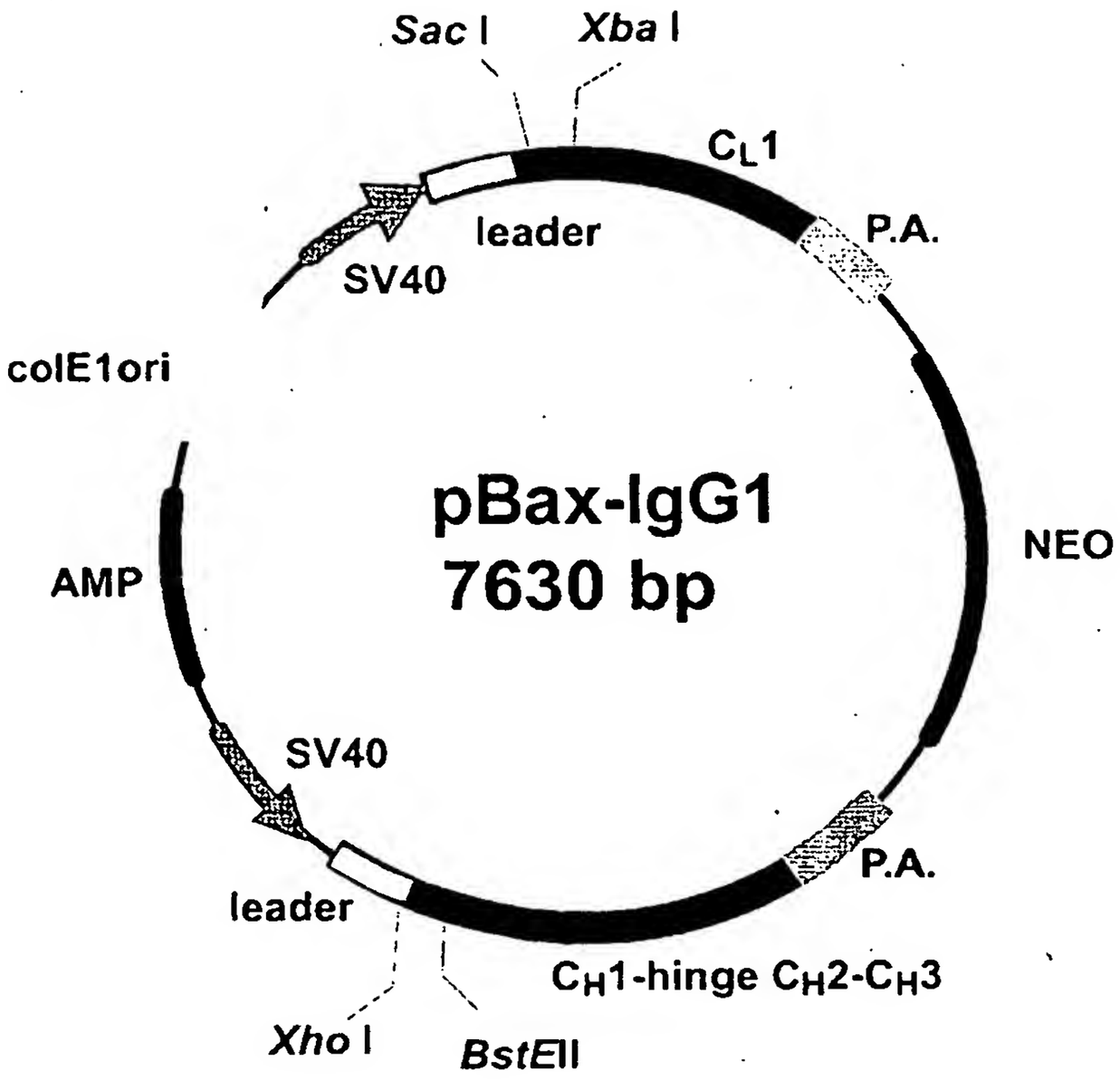
Sac I
Xba I
C_L1
leader
P.A.
SV40
colE1ori
pBax-IgG1
7630 bp
NEO
AMP
SV40
P.A.
leader
C_H1-hinge C_H2-C_H3
Xho I
BstEII

Figure 23

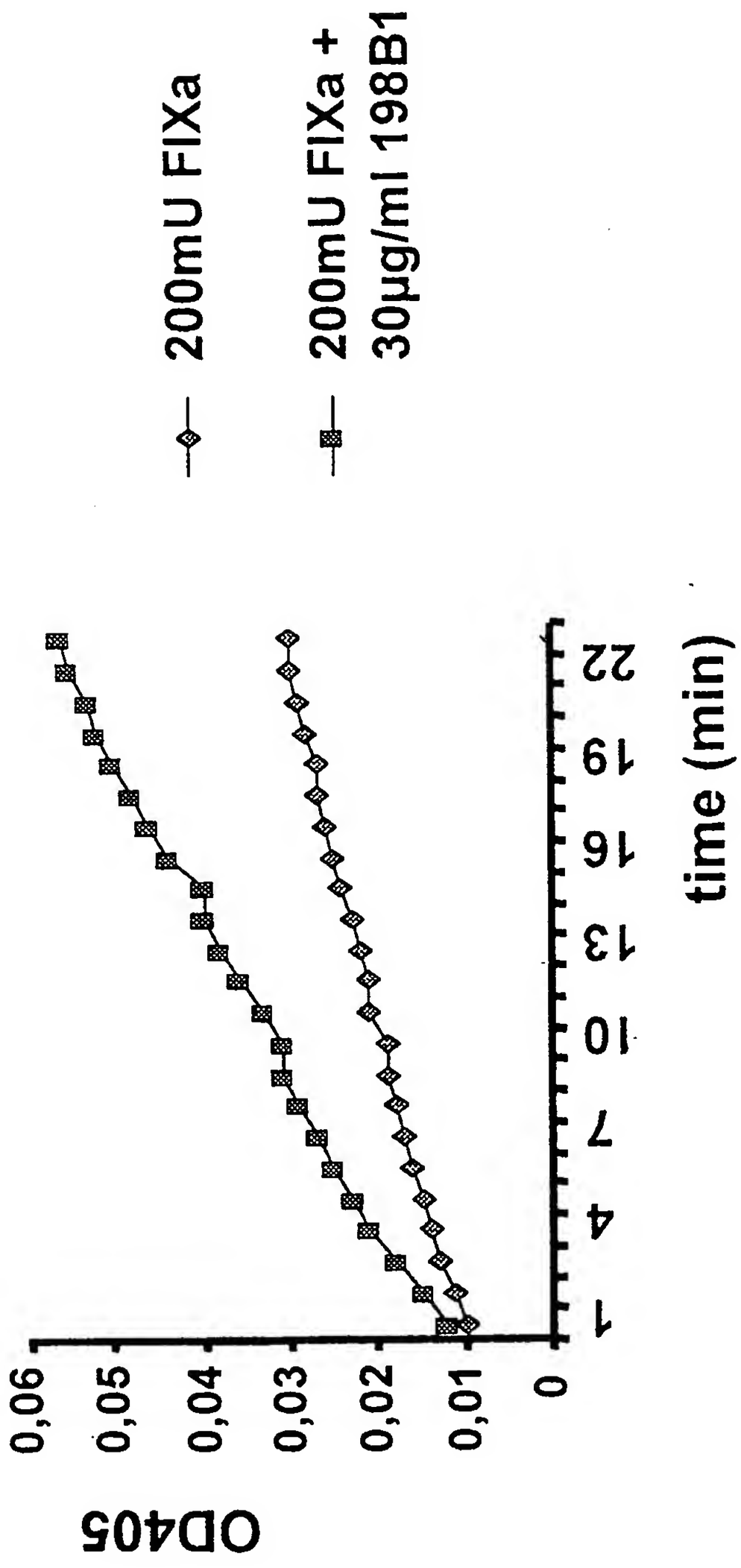
OD405
0,06
0,05
0,04
0,03
0,02
0,01
0
1
4
7
10
13
16
19
22
time (min)
200mU FIXa
200mU FIXa +
300µg/ml 198B1

Fig. 24A

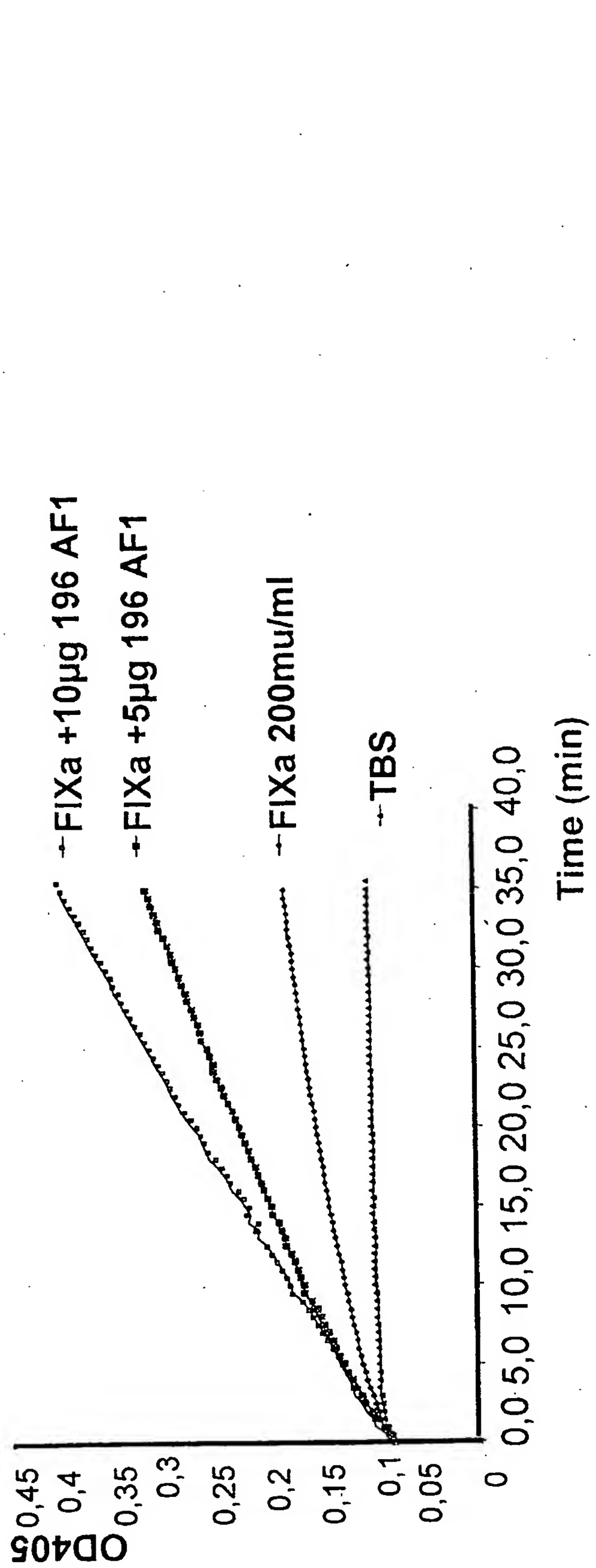
FIXa +10µg 196 AF1
FIXa +5µg 196 AF1
FIXa 200mu/ml
TBS
OD405
0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
0,0 5,0 10,0 15,0 20,0 25,0 30,0 35,0 40,0
Time (min)

Fig. 24B

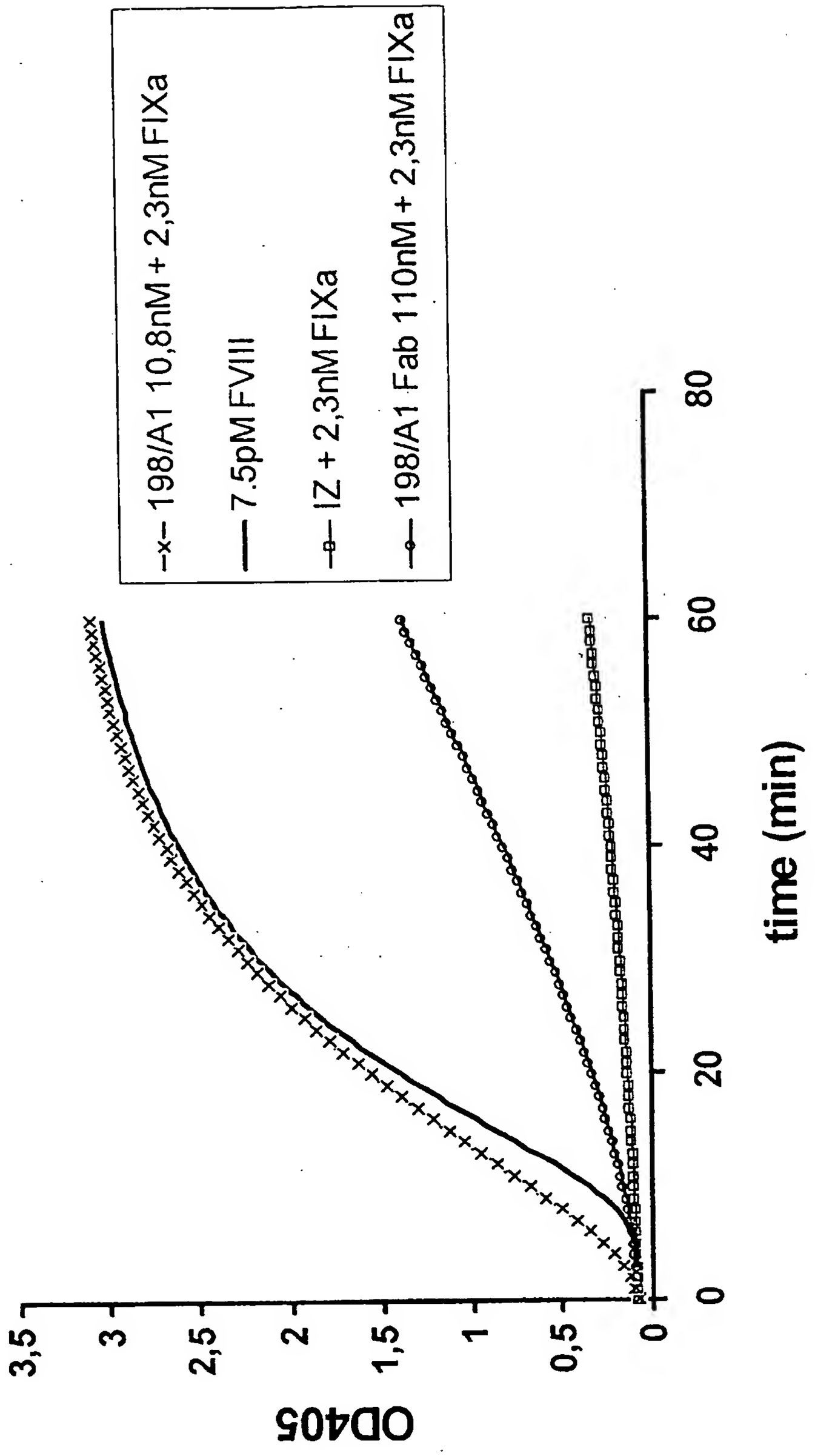
198/A1 10,8nM + 2,3nM FIXa
7.5pM FVIII
IZ + 2,3nM FIXa
198/A1 Fab 110nM + 2,3nM FIXa
OD405
3,5
3
2,5
2
1,5
1
0,5
0
0
20
40
60
80
time (min)

Fig. 25

PelB-leader

```
+1   M   K   Y   L   L   P   T   A   A   A   G   L   L   L
1   ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA
    TAC TTT ATG GAT AAC GGA TGC CGT CGG CGA CCT AAC AAT AAT

                                                VH
+1   L   A   A   Q   P   A   M   A | E   V   K   L   V   E
43  CTC GCG GCC CAG CCG GCC ATG GCG|GAG GTG AAG CTG GTG GAG
    GAG CGC CGG GTC GGC CGG TAC CGC|CTC CAC TTC GAC CAC CTC

+1   S   G   G   G   L   V   K   P   G   G   S   L   K   L
85  TCT GGG GGA GGC TTA GTG AAG CCT GGA GGG TCC CTG AAA CTC
    AGA CCC CCT CCG AAT CAC TTC GGA CCT CCC AGG GAC TTT GAG

+1   S   C   A   A   S   G   F   T   F   S   S   Y   T   M
127 TCC TGT GCA GCC TCT GGA TTC ACT TTC AGT AGC TAT ACC ATG
    AGG ACA CGT CGG AGA CCT AAG TGA AAG TCA TCG ATA TGG TAC

+1   S   W   V   R   Q   T   P   E   K   R   L   E   W   V
169 TCT TGG GTT CGC CAG ACT CCG GAG AAG AGG CTG GAG TGG GTC
    AGA ACC CAA GCG GTC TGA GGC CTC TTC TCC GAC CTC ACC CAG

+1   A   T   I   S   S   G   G   S   S   T   Y   Y   P   D
211 GCA ACC ATT AGT AGT GGN GGT AGT TCC ACC TAC TAT CCA GAC
    CGT TGG TAA TCA TCA CCN CCA TCA AGG TGG ATG ATA GGT CTG

+1   S   V   K   G   R   F   T   I   S   R   D   N   A   K
253 AGT GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG
    TCA CAC TTC CCG GCT AAG TGG TAG AGG TCT CTG TTA CGG TTC

+1   N   T   L   Y   L   Q   M   S   S   L   R   S   E   D
295 AAC ACC CTG TAC CTG CAA ATG AGC AGT CTG AGG TCT GAG GAC
    TTG TGG GAC ATG GAC GTT TAC TCG TCA GAC TCC AGA CTC CTG

+1   T   A   M   Y   Y   C   T   R   E   G   G   G   F   T
337 ACA GCC ATG TAT TAC TGT ACA AGA GAG GGG GGT GGT TTC ACC
    TGT CGG TAC ATA ATG ACA TGT TCT CTC CCC CCA CCA AAG TGG

+1   V   N   W   Y   F   D   V   W   G   A   G   T   S   V
379 GTC AAC TGG TAC TTC GAT GTC TGG GGC GCA GGA ACC TCA GTC
    CAG TTG ACC ATG AAG CTA CAG ACC CCG CGT CCT TGG AGT CAG

                                    Linker
+1   T   V   S   S | G   G   G   G   S   G   G   R   A   S
421 ACC GTC TCC TCA|GGT GGA GGC GGT TCA GGT GGG CGC GCC TCT
    TGG CAG AGG AGT|CCA CCT CCG CCA AGT CCA CCC GCG CGG AGA
```

Fig. 26-1

```
                                                  VL
  +1   G    G    G    G    S   D    I    V    L    T    Q    S    P    A
463   GGC  GGT  GGC  GGA  TCG GAC  ATT  GTG  CTG  ACA  CAG  TCT  CCA  GCT
      CCG  CCA  CCG  CCT  AGC CTG  TAA  CAC  GAC  TGT  GTC  AGA  GGT  CGA

  +1   S    L    A    V    S   L    G    Q    R    A    T    I    S    C
505   TCT  TTG  GCT  GTG  TCT CTA  GGG  CAG  AGG  GCC  ACC  ATA  TCC  TGC
      AGA  AAC  CGA  CAC  AGA GAT  CCC  GTC  TCC  CGG  TGG  TAT  AGG  ACG

  +1   R    A    S    E    S   V    D    S    Y    G    Y    N    F    M
547   AGA  GCC  AGT  GAA  AGT GTT  GAT  AGT  TAT  GGC  TAT  AAT  TTT  ATG
      TCT  CGG  TCA  CTT  TCA CAA  CTA  TCA  ATA  CCG  ATA  TTA  AAA  TAC

  +1   H    W    Y    Q    Q   I    P    G    Q    P    P    K    L    L
589   CAC  TGG  TAT  CAG  CAG ATA  CCA  GGA  CAG  CCA  CCC  AAA  CTC  CTC
      GTG  ACC  ATA  GTC  GTC TAT  GGT  CCT  GTC  GGT  GGG  TTT  GAG  GAG

  +1   I    Y    R    A    S   N    L    E    S    G    I    P    A    R
631   ATC  TAT  CGT  GCA  TCC AAC  CTA  GAG  TCT  GGG  ATC  CCT  GCC  AGG
      TAG  ATA  GCA  CGT  AGG TTG  GAT  CTC  AGA  CCC  TAG  GGA  CGG  TCC

  +1   F    S    G    S    G   S    R    T    D    F    T    L    T    I
673   TTC  AGT  GGC  AGT  GGG TCT  AGG  ACA  GAC  TTC  ACC  CTC  ACC  ATT
      AAG  TCA  CCG  TCA  CCC AGA  TCC  TGT  CTG  AAG  TGG  GAG  TGG  TAA

  +1   N    P    V    E    A   D    D    V    A    T    Y    Y    C    Q
715   AAT  CCT  GTG  GAG  GCT GAT  GAT  GTT  GCA  ACC  TAT  TAC  TGT  CAG
      TTA  GGA  CAC  CTC  CGA CTA  CTA  CAA  CGT  TGG  ATA  ATG  ACA  GTC

  +1   Q    S    N    E    D   P    L    T    F    G    T    G    T    R
757   CAA  AGT  AAT  GAG  GAT CCG  CTC  ACG  TTC  GGT  ACT  GGG  ACC  AGA
      GTT  TCA  TTA  CTC  CTA GGC  GAG  TGC  AAG  CCA  TGA  CCC  TGG  TCT

                                        Spacer          Alkaline phosphatase
  +1   L    E    I    K    R   A    A    A    A    R    A    P    E    M
799   CTG  GAA  ATA  AAA  CGG GCG  GCC  GCA  GCC  CGG  GCA  CCA  GAA  ATG
      GAC  CTT  TAT  TTT  GCC CGC  CGG  CGT  CGG  GCC  CGT  GGT  CTT  TAC

  +1   P    V    L    E    N   R    A    A    Q    G    D    I    T    A
841   CCT  GTT  CTG  GAA  AAC CGG  GCT  GCT  CAG  GGC  GAT  ATT  ACT  GCA
      GGA  CAA  GAC  CTT  TTG GCC  CGA  CGA  GTC  CCG  CTA  TAA  TGA  CGT

  +1   P    G    G    A    R   R    L    T    G    D    Q    T    A    A
883   CCC  GGC  GGT  GCT  CGC CGT  TTA  ACG  GGT  GAT  CAG  ACT  GCC  GCT
      GGG  CCG  CCA  CGA  GCG GCA  AAT  TGC  CCA  CTA  GTC  TGA  CGG  CGA

  +1   L    R    D    S    L   S    D    K    P    A    K    N    I    I
925   CTG  CGT  GAT  TCT  CTT AGC  GAT  AAA  CCT  GCA  AAA  AAT  ATT  ATT
      GAC  GCA  CTA  AGA  GAA TCG  CTA  TTT  GGA  CGT  TTT  TTA  TAA  TAA
```

Fig. 26-2

```
   +1  L   L   I   G   D   G   M   G   D   S   E   I   T   A
 967   TTG CTG ATT GGC GAT GGG ATG GGG GAC TCG GAA ATT ACT GCC
       AAC GAC TAA CCG CTA CCC TAC CCC CTG AGC CTT TAA TGA CGG

   +1  A   R   N   Y   A   E   G   A   G   G   F   F   K   G
1009   GCA CGT AAT TAT GCC GAA GGT GCG GGC GGC TTT TTT AAA GGT
       CGT GCA TTA ATA CGG CTT CCA CGC CCG CCG AAA AAA TTT CCA

   +1  I   D   A   L   P   L   T   G   Q   Y   T   H   Y   A
1051   ATA GAT GCC TTA CCG CTT ACC GGG CAA TAC ACT CAC TAT GCG
       TAT CTA CGG AAT GGC GAA TGG CCC GTT ATG TGA GTG ATA CGC

   +1  L   N   K   K   T   G   K   P   D   Y   V   T   D   S
1093   CTG AAT AAA AAA ACC GGC AAA CCG GAC TAC GTC ACC GAC TCG
       GAC TTA TTT TTT TGG CCG TTT GGC CTG ATG CAG TGG CTG AGC

   +1  A   A   S   A   T   A   W   S   T   G   V   K   T   Y
1135   GCT GCA TCA GCA ACC GCC TGG TCA ACC GGT GTC AAA ACC TAT
       CGA CGT AGT CGT TGG CGG ACC AGT TGG CCA CAG TTT TGG ATA

   +1  N   G   A   L   G   V   D   I   H   E   K   D   H   P
1177   AAC GGC GCG CTG GGC GTC GAT ATT CAC GAA AAA GAT CAC CCA
       TTG CCG CGC GAC CCG CAG CTA TAA GTG CTT TTT CTA GTG GGT

   +1  T   I   L   E   M   A   K   A   A   G   L   A   T   G
1219   ACG ATT CTG GAA ATG GCA AAA GCC GCA GGT CTG GCG ACC GGT
       TGC TAA GAC CTT TAC CGT TTT CGG CGT CCA GAC CGC TGG CCA

   +1  N   V   S   T   A   E   L   Q   D   A   T   P   A   A
1261   AAC GTT TCT ACC GCA GAG TTG CAG GAT GCC ACG CCC GCT GCG
       TTG CAA AGA TGG CGT CTC AAC GTC CTA CGG TGC GGG CGA CGC

   +1  L   V   A   H   V   T   S   R   K   C   Y   G   P   S
1303   CTG GTG GCA CAT GTG ACC TCG CGC AAA TGC TAC GGT CCG AGC
       GAC CAC CGT GTA CAC TGG AGC GCG TTT ACG ATG CCA GGC TCG

   +1  A   T   S   E   K   C   P   G   N   A   L   E   K   G
1345   GCG ACC AGT GAA AAA TGT CCG GGT AAC GCT CTG GAA AAA GGC
       CGC TGG TCA CTT TTT ACA GGC CCA TTG CGA GAC CTT TTT CCG

   +1  G   K   G   S   I   T   E   Q   L   L   N   A   R   A
1387   GGA AAA GGA TCG ATT ACC GAA CAG CTG CTT AAC GCT CGT GCC
       CCT TTT CCT AGC TAA TGG CTT GTC GAC GAA TTG CGA GCA CGG

   +1  D   V   T   L   G   G   G   A   K   T   F   A   E   T
1429   GAC GTT ACG CTT GGC GGC GGC GCA AAA ACC TTT GCT GAA ACG
       CTG CAA TGC GAA CCG CCG CCG CGT TTT TGG AAA CGA CTT TGC
```

Fig. 26-3

```
   +1  A   T   A   G   E   W   Q   G   K   T   L   R   E   Q
1471  GCA ACC GCT GGT GAA TGG CAG GGA AAA ACG CTG CGT GAA CAG
      CGT TGG CGA CCA CTT ACC GTC CCT TTT TGC GAC GCA CTT GTC

   +1  A   Q   A   R   G   Y   Q   L   V   S   D   A   A   S
1513  GCA CAG GCG CGT GGT TAT CAG TTG GTG AGC GAT GCT GCC TCA
      CGT GTC CGC GCA CCA ATA GTC AAC CAC TCG CTA CGA CGG AGT

   +1  L   N   S   V   T   E   A   N   Q   Q   K   P   L   L
1555  CTG AAT TCG GTG ACG GAA GCG AAT CAG CAA AAA CCC CTG CTT
      GAC TTA AGC CAC TGC CTT CGC TTA GTC GTT TTT GGG GAC GAA

   +1  G   L   F   A   D   G   N   M   P   V   R   W   L   G
1597  GGC CTG TTT GCT GAC GGC AAT ATG CCA GTG CGC TGG CTA GGA
      CCG GAC AAA CGA CTG CCG TTA TAC GGT CAC GCG ACC GAT CCT

   +1  P   K   A   T   Y   H   G   N   I   D   K   P   A   V
1639  CCG AAA GCA ACG TAC CAT GGC AAT ATC GAT AAG CCC GCA GTC
      GGC TTT CGT TGC ATG GTA CCG TTA TAG CTA TTC GGG CGT CAG

   +1  T   C   T   P   N   P   Q   R   N   D   S   V   P   T
1681  ACC TGT ACG CCA AAT CCG CAA CGT AAT GAC AGT GTA CCA ACC
      TGG ACA TGC GGT TTA GGC GTT GCA TTA CTG TCA CAT GGT TGG

   +1  L   A   Q   M   T   D   K   A   I   E   L   L   S   K
1723  CTG GCG CAG ATG ACC GAC AAA GCC ATT GAA TTG TTG AGT AAA
      GAC CGC GTC TAC TGG CTG TTT CGG TAA CTT AAC AAC TCA TTT

   +1  N   E   K   G   F   F   L   Q   V   E   G   A   S   I
1765  AAT GAG AAA GGC TTT TTC CTG CAA GTT GAA GGT GCG TCA ATC
      TTA CTC TTT CCG AAA AAG GAC GTT CAA CTT CCA CGC AGT TAG

   +1  D   K   Q   D   H   A   A   N   P   C   G   Q   I   G
1807  GAT AAA CAG GAT CAT GCT GCG AAT CCT TGT GGG CAA ATT GGC
      CTA TTT GTC CTA GTA CGA CGC TTA GGA ACA CCC GTT TAA CCG

   +1  E   T   V   D   L   D   E   A   V   Q   R   A   L   E
1849  GAG ACG GTC GAT CTC GAT GAA GCC GTA CAA CGG GCG CTG GAA
      CTC TGC CAG CTA GAG CTA CTT CGG CAT GTT GCC CGC GAC CTT

   +1  F   A   K   K   E   G   N   T   L   V   I   V   T   A
1891  TTC GCT AAA AAG GAG GGT AAC ACG CTG GTC ATA GTC ACC GCT
      AAG CGA TTT TTC CTC CCA TTG TGC GAC CAG TAT CAG TGG CGA

   +1  D   H   A   H   A   S   Q   I   V   A   P   D   T   K
1933  GAT CAC GCC CAC GCC AGC CAG ATT GTT GCG CCG GAT ACC AAA
      CTA GTG CGG GTG CGG TCG GTC TAA CAA CGC GGC CTA TGG TTT

   +1  A   P   G   L   T   Q   A   L   N   T   K   D   G   A
1975  GCT CCG GGC CTC ACC CAG GCG CTA AAT ACC AAA GAT GGC GCA
      CGA GGC CCG GAG TGG GTC CGC GAT TTA TGG TTT CTA CCG CGT
```

Fig. 26-4

```
   +1  V   M   V   M   S   Y   G   N   S   E   E   D   S   Q
2017  GTG ATG GTG ATG AGT TAC GGG AAC TCC GAA GAG GAT TCA CAA
      CAC TAC CAC TAC TCA ATG CCC TTG AGG CTT CTC CTA AGT GTT

   +1  E   H   T   G   S   Q   L   R   I   A   A   Y   G   P
2059  GAA CAT ACC GGC AGT CAG TTG CGT ATT GCG GCG TAT GGC CCG
      CTT GTA TGG CCG TCA GTC AAC GCA TAA CGC CGC ATA CCG GGC

   +1  H   A   A   N   V   V   G   L   T   D   Q   T   D   L
2101  CAT GCC GCC AAT GTT GTT GGA CTG ACC GAC CAG ACC GAT CTC
      GTA CGG CGG TTA CAA CAA CCT GAC TGG CTG GTC TGG CTA GAG

                                                  |His tag
   +1  F   Y   T   M   K   A   A   L   G   D   I | A   H   H
2143  TTC TAC ACC ATG AAA GCC GCT CTG GGG GAT ATC |GCA CAC CAT
      AAG ATG TGG TAC TTT CGG CGA GAC CCC CTA TAG |CGT GTG GTA

    +1  H   H   H   H   *
2185  CAC CAT CAC CAT TAA
      GTG GTA GTG GTA ATT
```

Fig. 26-5

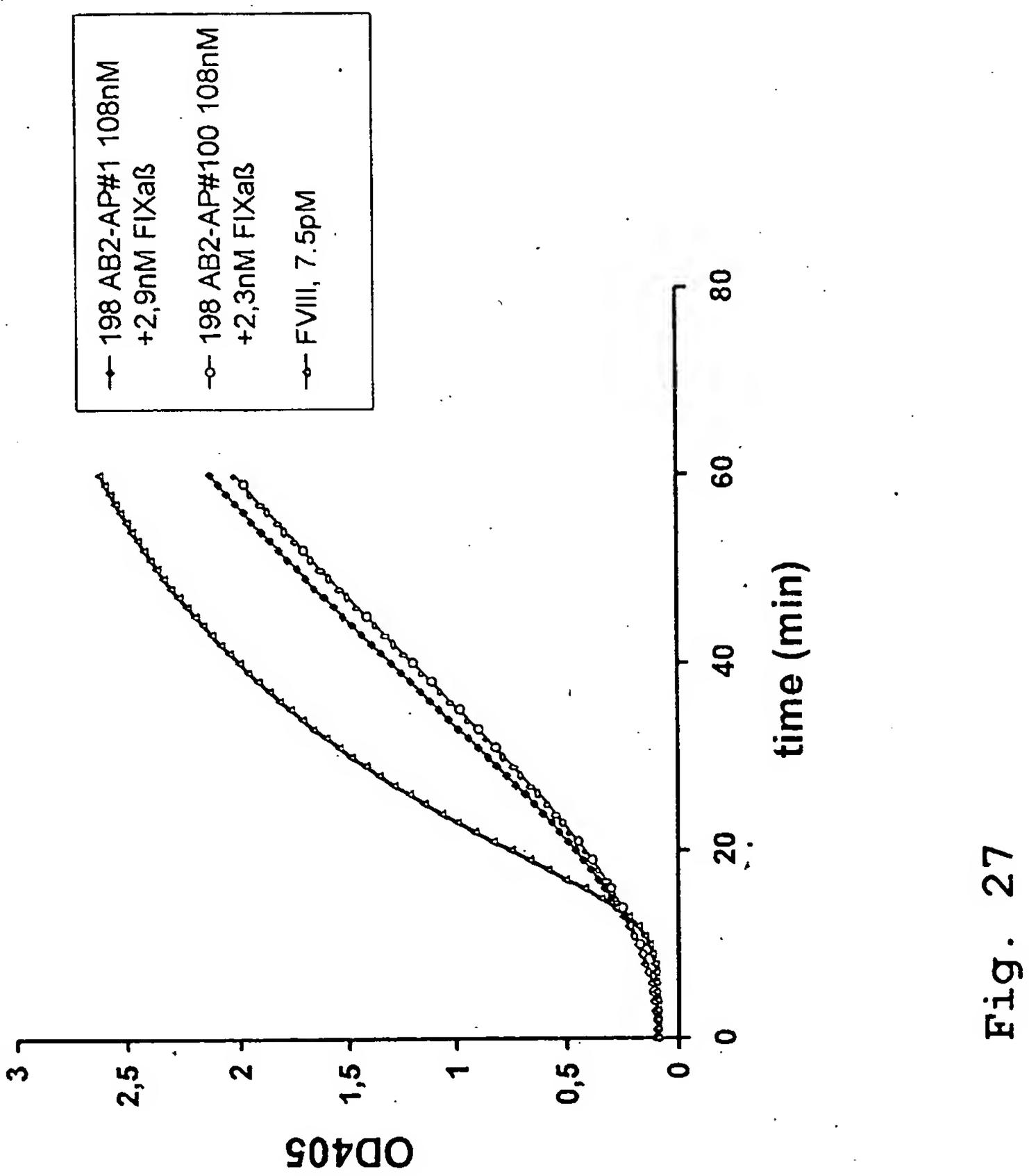
198 AB2-AP#1 108nM
+2,9nM FIXaß
198 AB2-AP#100 108nM
+2,3nM FIXaß
FVIII, 7.5pM
OD405
time (min)
0
0,5
1
1,5
2
2,5
3
20
40
60
80

Fig. 27

PelB-Leader

```
+1   M   K   Y   L   L   P   T   A   A   A   G   L   L   L   L
1   ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC
    TAC TTT ATG GAT AAC GGA TGC CGT CGG CGA CCT AAC AAT AAT GAG
```

VH

```
+1   A   A   Q   P   A   M   A   E   V   K   L   V   E   S   G
46  GCG GCC CAG CCG GCC ATG GCG GAG GTG AAG CTG GTG GAG TCT GGG
    CGC CGG GTC GGC CGG TAC CGC CTC CAC TTC GAC CAC CTC AGA CCC

+1   G   G   L   V   K   P   G   G   S   L   K   L   S   C   A
91  GGA GGC TTA GTG AAG CCT GGA GGG TCC CTG AAA CTC TCC TGT GCA
    CCT CCG AAT CAC TTC GGA CCT CCC AGG GAC TTT GAG AGG ACA CGT

+1   A   S   G   F   T   F   S   S   Y   T   M   S   W   V   R
136 GCC TCT GGA TTC ACT TTC AGT AGC TAT ACC ATG TCT TGG GTT CGC
    CGG AGA CCT AAG TGA AAG TCA TCG ATA TGG TAC AGA ACC CAA GCG

+1   Q   T   P   E   K   R   L   E   W   V   A   T   I   S   S
181 CAG ACT CCG GAG AAG AGG CTG GAG TGG GTC GCA ACC ATT AGT AGT
    GTC TGA GGC CTC TTC TCC GAC CTC ACC CAG CGT TGG TAA TCA TCA

+1   G   G   S   S   T   Y   Y   P   D   S   V   K   G   R   F
226 GGN GGT AGT TCC ACC TAC TAT CCA GAC AGT GTG AAG GGC CGA TTC
    CCN CCA TCA AGG TGG ATG ATA GGT CTG TCA CAC TTC CCG GCT AAG

+1   T   I   S   R   D   N   A   K   N   T   L   Y   L   Q   M
271 ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC CTG TAC CTG CAA ATG
    TGG TAG AGG TCT CTG TTA CGG TTC TTG TGG GAC ATG GAC GTT TAC

+1   S   S   L   R   S   E   D   T   A   M   Y   Y   C   T   R
316 AGC AGT CTG AGG TCT GAG GAC ACA GCC ATG TAT TAC TGT ACA AGA
    TCG TCA GAC TCC AGA CTC CTG TGT CGG TAC ATA ATG ACA TGT TCT

+1   E   G   G   G   F   T   V   N   W   Y   F   D   V   W   G
361 GAG GGG GGT GGT TTC ACC GTC AAC TGG TAC TTC GAT GTC TGG GGC
    CTC CCC CCA CCA AAG TGG CAG TTG ACC ATG AAG CTA CAG ACC CCG
```

Linker

```
+1   A   G   T   S   V   T   V   S   S   G   G   G   G   S   G
406 GCA GGA ACC TCA GTC ACC GTC TCC TCA GGT GGA GGC GGT TCA GGT
    CGT CCT TGG AGT CAG TGG CAG AGG AGT CCA CCT CCG CCA AGT CCA
```

VL

```
+1   G   R   A   S   G   G   G   G   S   D   I   V   L   T   Q
451 GGG CGC GCC TCT GGC GGT GGC GGA TCG GAC ATT GTG CTG ACA CAG
    CCC GCG CGG AGA CCG CCA CCG CCT AGC CTG TAA CAC GAC TGT GTC
```

Fig. 2P-1

```
  +1  X   P   A   S   L   A   V   S   L   G   Q   R   A   T   I
496  TNT CCA GCT TCT TTG GCT GTG TCT CTA GGG CAG AGG GCC ACC ATA
     ANA GGT CGA AGA AAC CGA CAC AGA GAT CCC GTC TCC CGG TGG TAT

  +1  S   C   R   A   S   E   S   V   D   S   Y   G   Y   N   F
541  TCN TGC AGA GCC AGT GAA AGT GTT GAT AGT TAT GGC TAT AAT TTT
     AGN ACG TCT CGG TCA CTT TCA CAA CTA TCA ATA CCG ATA TTA AAA

  +1  M   H   W   Y   Q   Q   I   P   G   Q   P   P   K   L   L
586  ATG CAC TGG TAT CAG CAG ATA CCA GGA CAG CCA CCC AAA CTC CTC
     TAC GTG ACC ATA GTC GTC TAT GGT CCT GTC GGT GGG TTT GAG GAG

  +1  I   Y   R   A   S   N   L   E   S   G   I   P   A   R   F
631  ATC TAT CGT GCA TCC AAC CTA GAG TCT GGG ATC CCT GCC AGG TTC
     TAG ATA GCA CGT AGG TTG GAT CTC AGA CCC TAG GGA CGG TCC AAG

  +1  S   G   S   G   S   R   T   D   F   T   L   T   I   N   P
676  AGT GGC AGT GGG TCT AGG ACA GAC TTC ACC CTC ACC ATT AAT CCT
     TCA CCG TCA CCC AGA TCC TGT CTG AAG TGG GAG TGG TAA TTA GGA

  +1  V   E   A   D   D   V   A   T   Y   Y   C   Q   Q   S   N
721  GTG GAG GCT GAT GAT GTT GCA ACC TAT TAC TGT CAG CAA AGT AAT
     CAC CTC CGA CTA CTA CAA CGT TGG ATA ATG ACA GTC GTT TCA TTA

  +1  E   D   P   L   T   F   G   T   G   T   R   L   E   I   K
766  GAG GAT CCG CTC ACG TTC GGT ACT GGG ACC AGA CTG GAA ATA AAA
     CTC CTA GGC GAG TGC AAG CCA TGA CCC TGG TCT GAC CTT TAT TTT

          Spacer      Hinge                                  Helix

  +1  R   A   A   A   P   K   P   S   T   P   P   G   S   S   R
811  CGG GCG GCC GCA CCG AAG CCT TCC ACT CCG CCC GGG TCT TCC CGT
     GCC CGC CGG CGT GGC TTC GGA AGG TGA GGC GGG CCC AGA AGG GCA

  +1  M   K   Q   L   E   D   K   V   E   E   L   L   S   K   N
856  ATG AAA CAG CTG GAA GAC AAA GTA GAG GAG CTC CTT AGC AAG AAC
     TAC TTT GTC GAC CTT CTG TTT CAT CTC CTC GAG GAA TCG TTC TTG

  +1  Y   H   L   E   N   E   V   A   R   L   K   K   L   V   G
901  TAC CAT CTA GAA AAC GAG GTA GCT CGT CTG AAA AAG CTT GTT GGT
     ATG GTA GAT CTT TTG CTC CAT CGA GCA GAC TTT TTC GAA CAA CCA

                Spacer      His-tag
  +1  E   R   G   G   H   H   H   H   H   H   *
946  GAA CGT GGT GGT CAC CAT CAC CAT CAC CAT TAA
     CTT GCA CCA CCA GTG GTA GTG GTA GTG GTA ATT
```

Fig. 28-2

PelB-leader

```
+1   M   K   Y   L   L   P   T   A   A   A   G   L   L   L
1   ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA
    TAC TTT ATG GAT AAC GGA TGC CGT CGG CGA CCT AAC AAT AAT
```

VH

```
+1   L   A   A   Q   P   A   M   A | E   V   Q   L   Q   Q
43  CTC GCG GCC CAG CCG GCC ATG GCC|GAG GTT CAG CTT CAG CAG
    GAG CGC CGG GTC GGC CGG TAC CGG|CTC CAA GTC GAA GTC GTC

+1   S   G   P   E   L   V   K   P   G   A   S   V   K   I
85  TCT GGA CCT GAG CTG GTG AAG CCC GGG GCC TCA GTG AAG ATT
    AGA CCT GGA CTC GAC CAC TTC GGG CCC CGG AGT CAC TTC TAA

+1   S   C   K   A   S   G   Y   A   F   S   S   S   W   M
127 TCC TGC AAA GCT TCT GGC TAC GCA TTC AGT AGC TCT TGG ATG
    AGG ACG TTT CGA AGA CCG ATG CGT AAG TCA TCG AGA ACC TAC

+1   N   W   V   K   Q   R   P   G   Q   G   L   E   W   I
169 AAC TGG GTG AAG CAG AGG CCT GGA CAG GGT CTT GAG TGG ATT
    TTG ACC CAC TTC GTC TCC GGA CCT GTC CCA GAA CTC ACC TAA

+1   G   R   I   Y   P   G   N   G   D   T   N   Y   N   G
211 GGA CGG ATT TAT CCT GGA AAT GGA GAT ACT AAC TAC AAT GGG
    CCT GCC TAA ATA GGA CCT TTA CCT CTA TGA TTG ATG TTA CCC

+1   K   F   K   G   K   A   T   L   T   A   D   K   S   S
253 AAG TTC AAG GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC TCC
    TTC AAG TTC CCG TTC CGG TGT GAC TGA CGT CTG TTT AGG AGG

+1   S   T   A   Y   M   Q   L   S   S   L   T   S   V   D
295 AGC ACA GCC TAC ATG CAG CTC AGC AGC CTG ACC TCT GTG GAC
    TCG TGT CGG ATG TAC GTC GAG TCG TCG GAC TGG AGA CAC CTG

+1   S   A   V   Y   F   C   A   D   G   N   V   Y   Y   Y
337 TCT GCG GTC TAT TTC TGT GCA GAT GGT AAC GTA TAT TAC TAT
    AGA CGC CAG ATA AAG ACA CGT CTA CCA TTG CAT ATA ATG ATA

+1   A   M   D   Y   W   G   Q   G   T   S   V   T   V   S
379 GCT ATG GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC
    CGA TAC CTG ATG ACC CCA GTT CCT TGG AGT CAG TGG CAG AGG
```

Linker

```
+1   S | G   G   G   G   S   G   G   R   A   S   G   G   G
421 TCA|GGT GGA GGC GGT TCA GGT GGG CGC GCC TCT GGC GGT GGC
    AGT|CCA CCT CCG CCA AGT CCA CCC GCG CGG AGA CCG CCA CCG
```

VL

```
+1   G   S | Q   I   V   L   T   Q   S   P   A   S   L   A
463 GGA TCG|CAA ATT GTT CTC ACC CAG TCT CCT GCT TCC TTA GCT
    CCT AGC|GTT TAA CAA GAG TGG GTC AGA GGA CGA AGG AAT CGA
```

Fig. 29-1

```
  +1  V   S   L   G   Q   R   A   T   I   S   C   R   A   S
505  GTA TCT CTG GGG CAG AGG GCC ACC ATC TCA TGC AGG GCC AGC
     CAT AGA GAC CCC GTC TCC CGG TGG TAG AGT ACG TCC CGG TCG

  +1  K   S   V   S   T   S   G   Y   S   Y   M   H   W   Y
547  AAA AGT GTC AGT ACA TCT GGC TAT AGT TAT ATG CAC TGG TAC
     TTT TCA CAG TCA TGT AGA CCG ATA TCA ATA TAC GTG ACC ATG

  +1  Q   Q   K   P   G   Q   P   P   K   L   L   I   Y   L
589  CAA CAG AAA CCA GGA CAG CCA CCC AAA CTC CTC ATC TAT CTT
     GTT GTC TTT GGT CCT GTC GGT GGG TTT GAG GAG TAG ATA GAA

  +1  A   S   N   L   E   S   G   V   P   A   R   F   S   G
631  GCA TCC AAC CTA GAA TCT GGG GTC CCT GCC AGG TTC AGT GGC
     CGT AGG TTG GAT CTT AGA CCC CAG GGA CGG TCC AAG TCA CCG

  +1  S   G   S   G   T   D   F   T   L   N   I   H   P   V
673  AGT GGG TCT GGG ACA GAC TTC ACC CTC AAC ATC CAT CCT GTG
     TCA CCC AGA CCC TGT CTG AAG TGG GAG TTG TAG GTA GGA CAC

  +1  E   E   E   D   A   A   T   Y   Y   C   Q   H   S   R
715  GAG GAG GAG GAT GCT GCA ACC TAT TAC TGT CAG CAC AGT AGG
     CTC CTC CTC CTA CGA CGT TGG ATA ATG ACA GTC GTG TCA TCC

  +1  E   L   P   R   T   F   G   G   G   T   K   L   E   I
757  GAG CTT CCT CGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC
     CTC GAA GGA GCC TGC AAG CCA CCT CCG TGG TTC GAC CTT TAG

                 |Spacer       |Alkaline phosphatase
  +1  K   R      |A   A   A   A|R   A   P   E   M   P   V   L
799  AAA CGG     |GCG GCC GCA GCC|CGG GCA CCA GAA ATG CCT GTT CTG
     TTT GCC     |CGC CGG CGT CGG|GCC CGT GGT CTT TAC GGA CAA GAC

  +1  E   N   R   A   A   Q   G   D   I   T   A   P   G   G
841  GAA AAC CGG GCT GCT CAG GGC GAT ATT ACT GCA CCC GGC GGT
     CTT TTG GCC CGA CGA GTC CCG CTA TAA TGA CGT GGG CCG CCA

  +1  A   R   R   L   T   G   D   Q   T   A   A   L   R   D
883  GCT CGC CGT TTA ACG GGT GAT CAG ACT GCC GCT CTG CGT GAT
     CGA GCG GCA AAT TGC CCA CTA GTC TGA CGG CGA GAC GCA CTA

  +1  S   L   S   D   K   P   A   K   N   I   I   L   L   I
925  TCT CTT AGC GAT AAA CCT GCA AAA AAT ATT ATT TTG CTG ATT
     AGA GAA TCG CTA TTT GGA CGT TTT TTA TAA TAA AAC GAC TAA

  +1  G   D   G   M   G   D   S   E   I   T   A   A   R   N
967  GGC GAT GGG ATG GGG GAC TCG GAA ATT ACT GCC GCA CGT AAT
     CCG CTA CCC TAC CCC CTG AGC CTT TAA TGA CGG CGT GCA TTA
```

Fig. 29-2

```
   +1  Y   A   E   G   A   G   G   F   F   K   G   I   D   A
1009  TAT GCC GAA GGT GCG GGC GGC TTT TTT AAA GGT ATA GAT GCC
      ATA CGG CTT CCA CGC CCG CCG AAA AAA TTT CCA TAT CTA CGG

   +1  L   P   L   T   G   Q   Y   T   H   Y   A   L   N   K
1051  TTA CCG CTT ACC GGG CAA TAC ACT CAC TAT GCG CTG AAT AAA
      AAT GGC GAA TGG CCC GTT ATG TGA GTG ATA CGC GAC TTA TTT

   +1  K   T   G   K   P   D   Y   V   T   D   S   A   A   S
1093  AAA ACC GGC AAA CCG GAC TAC GTC ACC GAC TCG GCT GCA TCA
      TTT TGG CCG TTT GGC CTG ATG CAG TGG CTG AGC CGA CGT AGT

   +1  A   T   A   W   S   T   G   V   K   T   Y   N   G   A
1135  GCA ACC GCC TGG TCA ACC GGT GTC AAA ACC TAT AAC GGC GCG
      CGT TGG CGG ACC AGT TGG CCA CAG TTT TGG ATA TTG CCG CGC

   +1  L   G   V   D   I   H   E   K   D   H   P   T   I   L
1177  CTG GGC GTC GAT ATT CAC GAA AAA GAT CAC CCA ACG ATT CTG
      GAC CCG CAG CTA TAA GTG CTT TTT CTA GTG GGT TGC TAA GAC

   +1  E   M   A   K   A   A   G   L   A   T   G   N   V   S
1219  GAA ATG GCA AAA GCC GCA GGT CTG GCG ACC GGT AAC GTT TCT
      CTT TAC CGT TTT CGG CGT CCA GAC CGC TGG CCA TTG CAA AGA

   +1  T   A   E   L   Q   D   A   T   P   A   A   L   V   A
1261  ACC GCA GAG TTG CAG GAT GCC ACG CCC GCT GCG CTG GTG GCA
      TGG CGT CTC AAC GTC CTA CGG TGC GGG CGA CGC GAC CAC CGT

   +1  H   V   T   S   R   K   C   Y   G   P   S   A   T   S
1303  CAT GTG ACC TCG CGC AAA TGC TAC GGT CCG AGC GCG ACC AGT
      GTA CAC TGG AGC GCG TTT ACG ATG CCA GGC TCG CGC TGG TCA

   +1  E   K   C   P   G   N   A   L   E   K   G   G   K   G
1345  GAA AAA TGT CCG GGT AAC GCT CTG GAA AAA GGC GGA AAA GGA
      CTT TTT ACA GGC CCA TTG CGA GAC CTT TTT CCG CCT TTT CCT

   +1  S   I   T   E   Q   L   L   N   A   R   A   D   V   T
1387  TCG ATT ACC GAA CAG CTG CTT AAC GCT CGT GCC GAC GTT ACG
      AGC TAA TGG CTT GTC GAC GAA TTG CGA GCA CGG CTG CAA TGC

   +1  L   G   G   G   A   K   T   F   A   E   T   A   T   A
1429  CTT GGC GGC GGC GCA AAA ACC TTT GCT GAA ACG GCA ACC GCT
      GAA CCG CCG CCG CGT TTT TGG AAA CGA CTT TGC CGT TGG CGA

   +1  G   E   W   Q   G   K   T   L   R   E   Q   A   Q   A
1471  GGT GAA TGG CAG GGA AAA ACG CTG CGT GAA CAG GCA CAG GCG
      CCA CTT ACC GTC CCT TTT TGC GAC GCA CTT GTC CGT GTC CGC
```

Fig. 29-3

```
   +1  R   G   Y   Q   L   V   S   D   A   A   S   L   N   S
1513  CGT GGT TAT CAG TTG GTG AGC GAT GCT GCC TCA CTG AAT TCG
      GCA CCA ATA GTC AAC CAC TCG CTA CGA CGG AGT GAC TTA AGC

   +1  V   T   E   A   N   Q   Q   K   P   L   L   G   L   F
1555  GTG ACG GAA GCG AAT CAG CAA AAA CCC CTG CTT GGC CTG TTT
      CAC TGC CTT CGC TTA GTC GTT TTT GGG GAC GAA CCG GAC AAA

   +1  A   D   G   N   M   P   V   R   W   L   G   P   K   A
1597  GCT GAC GGC AAT ATG CCA GTG CGC TGG CTA GGA CCG AAA GCA
      CGA CTG CCG TTA TAC GGT CAC GCG ACC GAT CCT GGC TTT CGT

   +1  T   Y   H   G   N   I   D   K   P   A   V   T   C   T
1639  ACG TAC CAT GGC AAT ATC GAT AAG CCC GCA GTC ACC TGT ACG
      TGC ATG GTA CCG TTA TAG CTA TTC GGG CGT CAG TGG ACA TGC

   +1  P   N   P   Q   R   N   D   S   V   P   T   L   A   Q
1681  CCA AAT CCG CAA CGT AAT GAC AGT GTA CCA ACC CTG GCG CAG
      GGT TTA GGC GTT GCA TTA CTG TCA CAT GGT TGG GAC CGC GTC

   +1  M   T   D   K   A   I   E   L   L   S   K   N   E   K
1723  ATG ACC GAC AAA GCC ATT GAA TTG TTG AGT AAA AAT GAG AAA
      TAC TGG CTG TTT CGG TAA CTT AAC AAC TCA TTT TTA CTC TTT

   +1  G   F   F   L   Q   V   E   G   A   S   I   D   K   Q
1765  GGC TTT TTC CTG CAA GTT GAA GGT GCG TCA ATC GAT AAA CAG
      CCG AAA AAG GAC GTT CAA CTT CCA CGC AGT TAG CTA TTT GTC

   +1  D   H   A   A   N   P   C   G   Q   I   G   E   T   V
1807  GAT CAT GCT GCG AAT CCT TGT GGG CAA ATT GGC GAG ACG GTC
      CTA GTA CGA CGC TTA GGA ACA CCC GTT TAA CCG CTC TGC CAG

   +1  D   L   D   E   A   V   Q   R   A   L   E   F   A   K
1849  GAT CTC GAT GAA GCC GTA CAA CGG GCG CTG GAA TTC GCT AAA
      CTA GAG CTA CTT CGG CAT GTT GCC CGC GAC CTT AAG CGA TTT

   +1  K   E   G   N   T   L   V   I   V   T   A   D   H   A
1891  AAG GAG GGT AAC ACG CTG GTC ATA GTC ACC GCT GAT CAC GCC
      TTC CTC CCA TTG TGC GAC CAG TAT CAG TGG CGA CTA GTG CGG

   +1  H   A   S   Q   I   V   A   P   D   T   K   A   P   G
1933  CAC GCC AGC CAG ATT GTT GCG CCG GAT ACC AAA GCT CCG GGC
      GTG CGG TCG GTC TAA CAA CGC GGC CTA TGG TTT CGA GGC CCG

   +1  L   T   Q   A   L   N   T   K   D   G   A   V   M   V
1975  CTC ACC CAG GCG CTA AAT ACC AAA GAT GGC GCA GTG ATG GTG
      GAG TGG GTC CGC GAT TTA TGG TTT CTA CCG CGT CAC TAC CAC
```

Fig. 29-4

```
   +1  M   S   Y   G   N   S   E   E   D   S   Q   E   H   T
2017  ATG AGT TAC GGG AAC TCC GAA GAG GAT TCA CAA GAA CAT ACC
      TAC TCA ATG CCC TTG AGG CTT CTC CTA AGT GTT CTT GTA TGG

   +1  G   S   Q   L   R   I   A   A   Y   G   P   H   A   A
2059  GGC AGT CAG TTG CGT ATT GCG GCG TAT GGC CCG CAT GCC GCC
      CCG TCA GTC AAC GCA TAA CGC CGC ATA CCG GGC GTA CGG CGG

   +1  N   V   V   G   L   T   D   Q   T   D   L   F   Y   T
2101  AAT GTT GTT GGA CTG ACC GAC CAG ACC GAT CTC TTC TAC ACC
      TTA CAA CAA CCT GAC TGG CTG GTC TGG CTA GAG AAG ATG TGG

                                                  His tag
   +1  M   K   A   A   L   G   D   I   A  |H   H   H   H   H
2143  ATG AAA GCC GCT CTG GGG GAT ATC GCA CAC CAT CAC CAT CAC
      TAC TTT CGG CGA GAC CCC CTA TAG CGT GTG GTA GTG GTA GTG

   +1  H   *
2185  CAT TAA
      GTA ATT
```

Fig. 29-5

PelB-Leader

```
+1   M   K   Y   L   L   P   T   A   A   A   G   L   L   L   L
1  ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC
   TAC TTT ATG GAT AAC GGA TGC CGT CGG CGA CCT AAC AAT AAT GAG
```

VH

```
+1   A   A   Q   P   A   M   A | E   V   Q   L   Q   Q   S   G
46 GCG GCC CAG CCG GCC ATG GCG |GAG GTT CAG CTT CAG CAG TCT GGA
   CGC CGG GTC GGC CGG TAC CGC |CTC CAA GTC GAA GTC GTC AGA CCT
```

```
+1   P   E   L   V   K   P   G   A   S   V   K   I   S   C   K
91 CCT GAG CTG GTG AAG CCC GGG GCC TCA GTG AAG ATT TCC TGC AAA
   GGA CTC GAC CAC TTC GGG CCC CGG AGT CAC TTC TAA AGG ACG TTT

+1   A   S   G   Y   A   F   S   S   S   W   M   N   W   V   K
136 GCT TCT GGC TAC GCA TTC AGT AGC TCT TGG ATG AAC TGG GTG AAG
    CGA AGA CCG ATG CGT AAG TCA TCG AGA ACC TAC TTG ACC CAC TTC

+1   Q   R   P   G   Q   G   L   E   W   I   G   R   I   Y   P
181 CAG AGG CCT GGA CAG GGT CTT GAG TGG ATT GGA CGG ATT TAT CCT
    GTC TCC GGA CCT GTC CCA GAA CTC ACC TAA CCT GCC TAA ATA GGA

+1   G   N   G   D   T   N   Y   N   G   K   F   K   G   K   A
226 GGA AAT GGA GAT ACT AAC TAC AAT GGG AAG TTC AAG GGC AAG GCC
    CCT TTA CCT CTA TGA TTG ATG TTA CCC TTC AAG TTC CCG TTC CGG

+1   T   L   T   A   D   K   S   S   S   T   A   Y   M   Q   L
271 ACA CTG ACT GCA GAC AAA TCC TCC AGC ACA GCC TAC ATG CAG CTC
    TGT GAC TGA CGT CTG TTT AGG AGG TCG TGT CGG ATG TAC GTC GAG

+1   S   S   L   T   S   V   D   S   A   V   Y   F   C   A   D
316 AGC AGC CTG ACC TCT GTG GAC TCT GCG GTC TAT TTC TGT GCA GAT
    TCG TCG GAC TGG AGA CAC CTG AGA CGC CAG ATA AAG ACA CGT CTA

+1   G   N   V   Y   Y   Y   A   M   D   Y   W   G   Q   G   T
361 GGT AAC GTA TAT TAC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC
    CCA TTG CAT ATA ATG ATA CGA TAC CTG ATG ACC CCA GTT CCT TGG
```

Linker

```
+1   S   V   T   V   S   S | G   G   G   G   S   G   G   R   A
406 TCA GTC ACC GTC TCC TCA |GGT GGA GGC GGT TCA GGT GGG CGC GCC
    AGT CAG TGG CAG AGG AGT |CCA CCT CCG CCA AGT CCA CCC GCG CGG
```

Fig. 30-1

```
                                        |  VL
  +1   S    G    G    G    G    S    |  Q    I    V    L    T    Q    S    P    A
451   TCT  GGC  GGT  GGC  GGA  TCG   |  CAA  ATT  GTT  CTC  ACC  CAG  TCT  CCT  GCT
      AGA  CCG  CCA  CCG  CCT  AGC   |  GTT  TAA  CAA  GAG  TGG  GTC  AGA  GGA  CGA

  +1   S    L    A    V    S    L    G    Q    R    A    T    I    S    C    R
496   TCC  TTA  GCT  GTA  TCT  CTG  GGG  CAG  AGG  GCC  ACC  ATC  TCA  TGC  AGG
      AGG  AAT  CGA  CAT  AGA  GAC  CCC  GTC  TCC  CGG  TGG  TAG  AGT  ACG  TCC

  +1   A    S    K    S    V    S    T    S    G    Y    S    Y    M    H    W
541   GCC  AGC  AAA  AGT  GTC  AGT  ACA  TCT  GGC  TAT  AGT  TAT  ATG  CAC  TGG
      CGG  TCG  TTT  TCA  CAG  TCA  TGT  AGA  CCG  ATA  TCA  ATA  TAC  GTG  ACC

  +1   Y    Q    Q    K    P    G    Q    P    P    K    L    L    I    Y    L
586   TAC  CAA  CAG  AAA  CCA  GGA  CAG  CCA  CCC  AAA  CTC  CTC  ATC  TAT  CTT
      ATG  GTT  GTC  TTT  GGT  CCT  GTC  GGT  GGG  TTT  GAG  GAG  TAG  ATA  GAA

  +1   A    S    N    L    E    S    G    V    P    A    R    F    S    G    S
631   GCA  TCC  AAC  CTA  GAA  TCT  GGG  GTC  CCT  GCC  AGG  TTC  AGT  GGC  AGT
      CGT  AGG  TTG  GAT  CTT  AGA  CCC  CAG  GGA  CGG  TCC  AAG  TCA  CCG  TCA

  +1   G    S    G    T    D    F    T    L    N    I    H    P    V    E    E
676   GGG  TCT  GGG  ACA  GAC  TTC  ACC  CTC  AAC  ATC  CAT  CCT  GTG  GAG  GAG
      CCC  AGA  CCC  TGT  CTG  AAG  TGG  GAG  TTG  TAG  GTA  GGA  CAC  CTC  CTC

  +1   E    D    A    A    T    Y    Y    C    Q    H    S    R    E    L    P
721   GAG  GAT  GCT  GCA  ACC  TAT  TAC  TGT  CAG  CAC  AGT  AGG  GAG  CTT  CCT
      CTC  CTA  CGA  CGT  TGG  ATA  ATG  ACA  GTC  GTG  TCA  TCC  CTC  GAA  GGA

                                                                  |  Spacer
  +1   R    T    F    G    G    G    T    K    L    E    I    K    R  |  A    A
766   CGG  ACG  TTC  GGT  GGA  GGC  ACC  AAG  CTG  GAA  ATC  AAA  CGG  |  GCG  GCC
      GCC  TGC  AAG  CCA  CCT  CCG  TGG  TTC  GAC  CTT  TAG  TTT  GCC  |  CGC  CGG

        |  Hinge                                                    |  Helix
  +1  A |  P    K    P    S    T    P    P    G    S    S    |  R    M    K    Q
811   GCA | CCG  AAG  CCT  TCC  ACT  CCG  CCC  GGG  TCT  TCC  |  CGT  ATG  AAA  CAG
      CGT | GGC  TTC  GGA  AGG  TGA  GGC  GGG  CCC  AGA  AGG  |  GCA  TAC  TTT  GTC

  +1   L    E    D    K    V    E    E    L    L    S    K    N    Y    H    L
856   CTG  GAA  GAC  AAA  GTA  GAG  GAG  CTC  CTT  AGC  AAG  AAC  TAC  CAT  CTA
      GAC  CTT  CTG  TTT  CAT  CTC  CTC  GAG  GAA  TCG  TTC  TTG  ATG  GTA  GAT

  +1   E    N    E    V    A    R    L    K    K    L    V    G    E    R  |  G
901   GAA  AAC  GAG  GTA  GCT  CGT  CTG  AAA  AAG  CTT  GTT  GGT  GAA  CGT  |  GGT
      CTT  TTG  CTC  CAT  CGA  GCA  GAC  TTT  TTC  GAA  CAA  CCA  CTT  GCA  |  CCA
```

Fig. 30-2

	Spacer	His-tag						
	+1	G	H	H	H	H	H	H	*
946	GGT		CAC	CAT	CAC	CAT	CAC	CAT	TAA
	CCA		GTG	GTA	GTG	GTA	GTG	GTA	ATT

Fig. 30-3

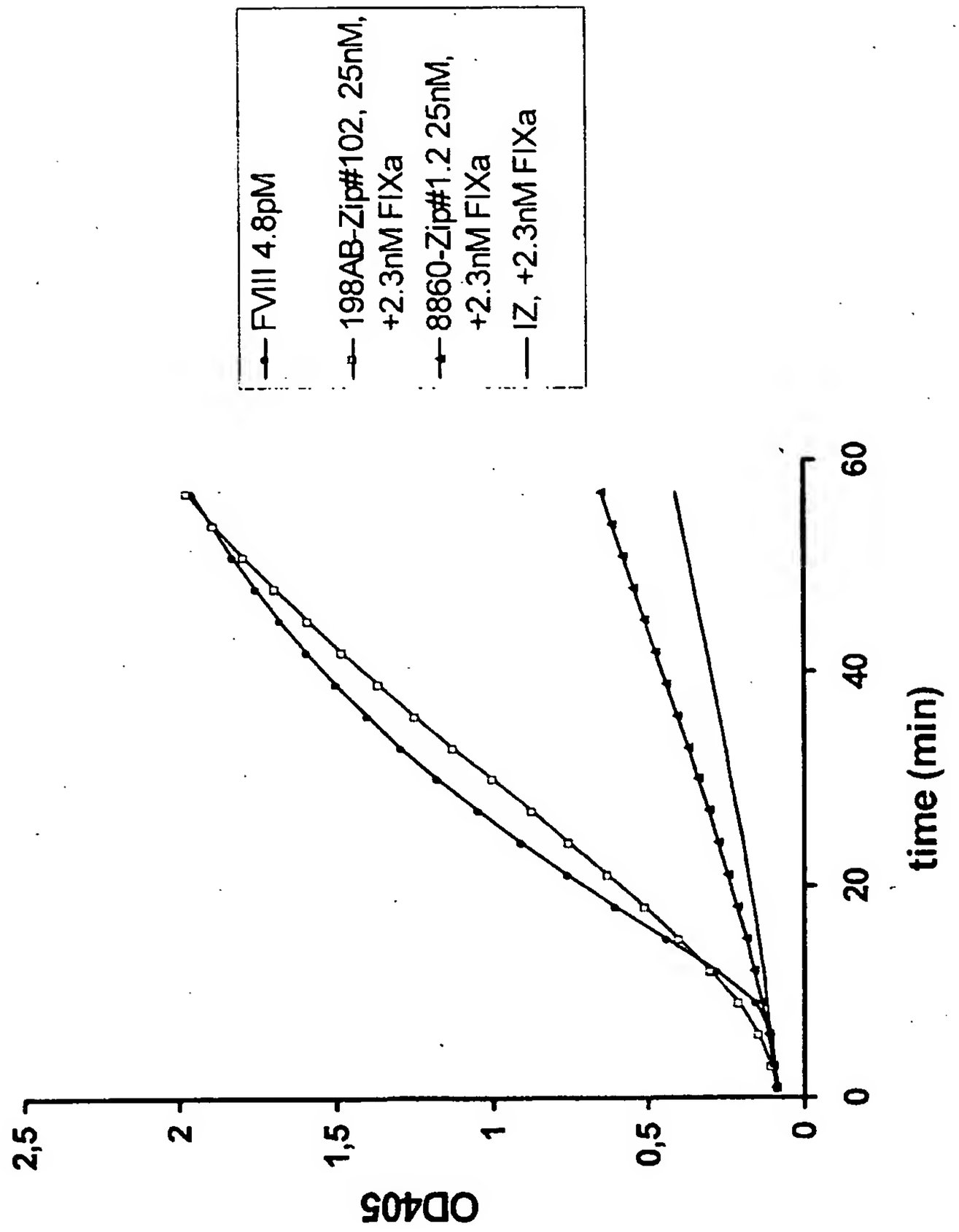
FVIII 4.8pM
198AB-Zip#102, 25nM, +2.3nM FIXa
8860-Zip#1.2 25nM +2.3nM FIXa
IZ, +2.3nM FIXa
OD405
2,5
2
1,5
1
0,5
0
0
20
40
60
time (min)

Fig. 31

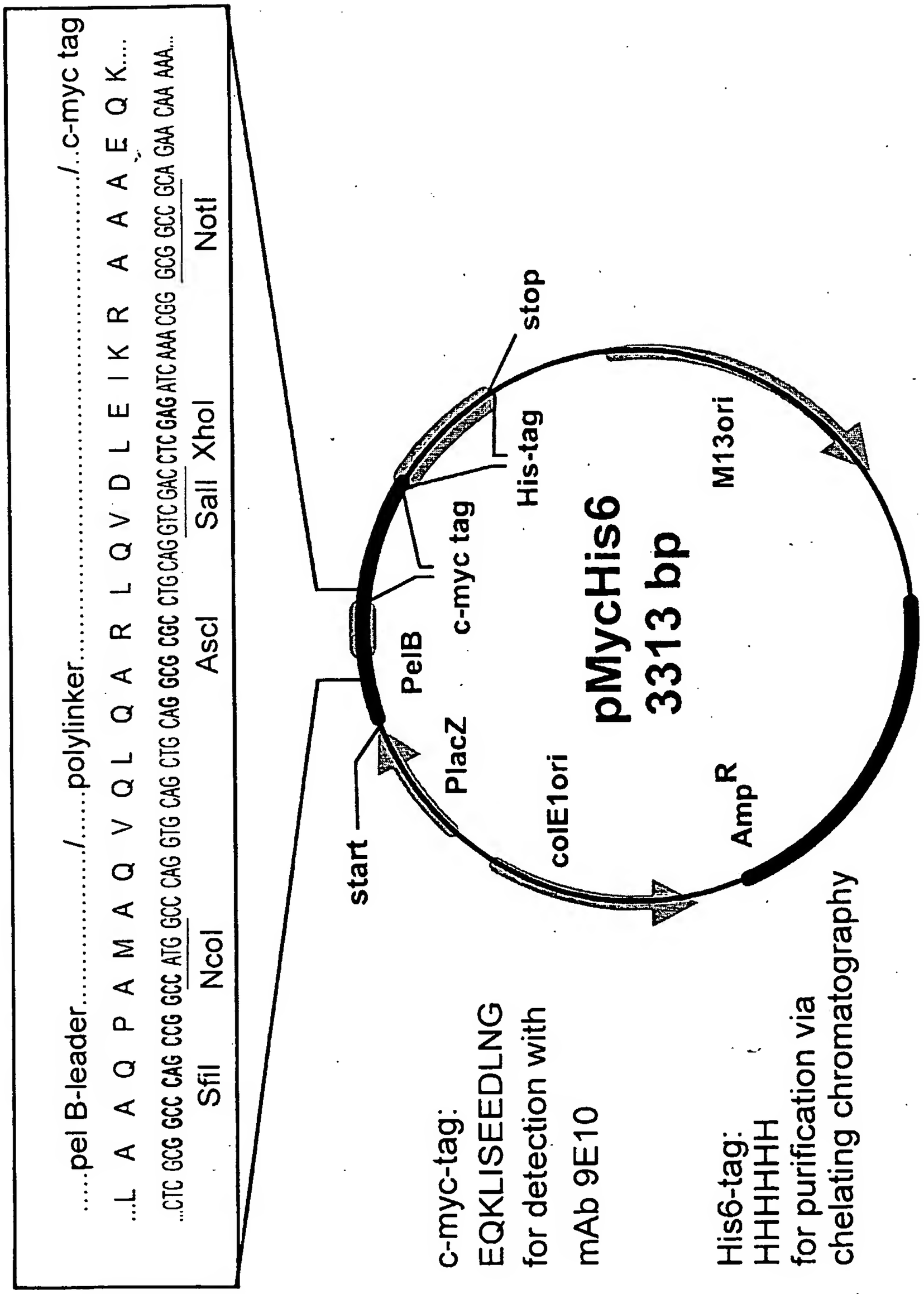
pMycHis6
3313 bp
start
PelB
c-myc tag
His-tag
stop
PlacZ
colE1ori
Amp^R
M13ori
pel B-leader.....
..L A A Q P A M A Q V Q L Q A R L Q V D L E I K R
...CTC GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG CAG GCG CGC CTG CAG GTC GAC CTC GAG ATC AAA CGG
SfiI NcoI AscI SalI XhoI
/.....polylinker.....
/..c-myc tag
A A A E Q K....
CGG GCC GCA GAA CAA AAA...
NotI
c-myc-tag:
EQKLISEEDLNG
for detection with
mAb 9E10
His6-tag:
HHHHHH
for purification via
chelating chromatography

Fig. 32

HindIII

2206 CAG GAA ACA GCT ATG ACC ATG ATT ACG CCA AGC TTC CAT GAA AAT
GTC CTT TGT CGA TAC TGG TAC TAA TGC GGT TCG AAG GTA CTT TTA

PelB-Leader

M K Y L L P T

2251 TCT ATT TCA AGG AGA CAG TCA TAA TGA AAT ACC TAT TGC CTA CGG
AGA TAA AGT TCC TCT GTC AGT ATT ACT TTA TGG ATA ACG GAT GCC

A A A G L L L L A A Q P A M A

SfiI

2296 CAG CCG CTG GAT TGT TAT TAC TCG CGG CCC AGC CGG CCA TGG CCC
GTC GGC GAC CTA ACA ATA ATG AGC GCC GGG TCG GCC GGT ACC GGG

Polylinker

Q V Q L Q A R L Q V D L E I K

AscI

2341 AGG TGC AGC TGC AGG CGC GCC TGC AGG TCG ACC TCG AGA TCA AAC
TCC ACG TCG ACG TCC GCG CGG ACG TCC AGC TGG AGC TCT AGT TTG

Spacer | Myc-tag

R | A A A | E Q K L I S E E D L N

NotI

2386 GGG CGG CCG CAG AAC AAA AAC TCA TCT CAG AAG AGG ATC TGA ATG
CCC GCC GGC GTC TTG TTT TTG AGT AGA GTC TTC TCC TAG ACT TAC

Spacer | His tag

G | A A | H H H H H H * *

EcoRI

2431 GGG CGG CAC ATC ACC ATC ACC ATC ACT AAT AAG AAT TCA CTG GCC
CCC GCC GTG TAG TGG TAG TGG TAG TGA TTA TTC TTA AGT GAC CGG

Fig. 33

PelB-leader

```
+1   M   K   Y   L   L   P   T   A   A   A   G   L   L   L   L
1   ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC
    TAC TTT ATG GAT AAC GGA TGC CGT CGG CGA CCT AAC AAT AAT GAG

                                                  | VH
+1   A   A   Q   P   A   M   A | E   V   K   L   V   E   S   G
46  GCG GCC CAG CCG GCC ATG GCC|GAG GTG AAG CTG GTG GAG TCT GGG
    CGC CGG GTC GGC CGG TAC CGG|CTC CAC TTC GAC CAC CTC AGA CCC

+1   G   G   L   V   K   P   G   G   S   L   K   L   S   C   A
91  GGA GGC TTA GTG AAG CCT GGA GGG TCC CTG AAA CTC TCC TGT GCA
    CCT CCG AAT CAC TTC GGA CCT CCC AGG GAC TTT GAG AGG ACA CGT

+1   A   S   G   F   T   F   S   S   Y   T   M   S   W   V   R
136 GCC TCT GGA TTC ACT TTC AGT AGC TAT ACC ATG TCT TGG GTT CGC
    CGG AGA CCT AAG TGA AAG TCA TCG ATA TGG TAC AGA ACC CAA GCG

+1   Q   T   P   E   K   R   L   E   W   V   A   T   I   S   S
181 CAG ACT CCG GAG AAG AGG CTG GAG TGG GTC GCA ACC ATT AGT AGT
    GTC TGA GGC CTC TTC TCC GAC CTC ACC CAG CGT TGG TAA TCA TCA

+1   G   G   S   S   T   Y   Y   P   D   S   V   K   G   R   F
226 GGN GGT AGT TCC ACC TAC TAT CCA GAC AGT GTG AAG GGC CGA TTC
    CCN CCA TCA AGG TGG ATG ATA GGT CTG TCA CAC TTC CCG GCT AAG

+1   T   I   S   R   D   N   A   K   N   T   L   Y   L   Q   M
271 ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC CTG TAC CTG CAA ATG
    TGG TAG AGG TCT CTG TTA CGG TTC TTG TGG GAC ATG GAC GTT TAC

+1   S   S   L   R   S   E   D   T   A   M   Y   Y   C   T   R
316 AGC AGT CTG AGG TCT GAG GAC ACA GCC ATG TAT TAC TGT ACA AGA
    TCG TCA GAC TCC AGA CTC CTG TGT CGG TAC ATA ATG ACA TGT TCT

+1   E   G   G   G   F   T   V   N   W   Y   F   D   V   W   G
361 GAG GGG GGT GGT TTC ACC GTC AAC TGG TAC TTC GAT GTC TGG GGC
    CTC CCC CCA CCA AAG TGG CAG TTG ACC ATG AAG CTA CAG ACC CCG

                                                  | Leader
+1   A   G   T   S   V   T   V   S   S | G   G   G   G   S   G
406 GCA GGA ACC TCA GTC ACC GTC TCC TCA|GGT GGA GGC GGT TCA GGT
    CGT CCT TGG AGT CAG TGG CAG AGG AGT|CCA CCT CCG CCA AGT CCA

                                                  | VK
+1   G   R   A   S   G   G   G   G   S | D   I   V   L   T   Q
451 GGG CGC GCC TCT GGC GGT GGC GGA TCG|GAC ATT GTG CTG ACA CAG
```

Fig. 34-1

```
     CCC GCG CGG AGA CCG CCA CCG CCT AGC CTG TAA CAC GAC TGT GTC

  +1  S   P   A   S   L   A   V   S   L   G   Q   R   A   T   I
496  TCT CCA GCT TCT TTG GCT GTG TCT CTA GGG CAG AGG GCC ACC ATA
     AGA GGT CGA AGA AAC CGA CAC AGA GAT CCC GTC TCC CGG TGG TAT

  +1  S   C   R   A   S   E   S   V   D   S   Y   G   Y   N   F
541  TCC TGC AGA GCC AGT GAA AGT GTT GAT AGT TAT GGC TAT AAT TTT
     AGG ACG TCT CGG TCA CTT TCA CAA CTA TCA ATA CCG ATA TTA AAA

  +1  M   H   W   Y   Q   Q   I   P   G   Q   P   P   K   L   L
586  ATG CAC TGG TAT CAG CAG ATA CCA GGA CAG CCA CCC AAA CTC CTC
     TAC GTG ACC ATA GTC GTC TAT GGT CCT GTC GGT GGG TTT GAG GAG

  +1  I   Y   R   A   S   N   L   E   S   G   I   P   A   R   F
631  ATC TAT CGT GCA TCC AAC CTA GAG TCT GGG ATC CCT GCC AGG TTC
     TAG ATA GCA CGT AGG TTG GAT CTC AGA CCC TAG GGA CGG TCC AAG

  +1  S   G   S   G   S   R   T   D   F   T   L   T   I   N   P
676  AGT GGC AGT GGG TCT AGG ACA GAC TTC ACC CTC ACC ATT AAT CCT
     TCA CCG TCA CCC AGA TCC TGT CTG AAG TGG GAG TGG TAA TTA GGA

  +1  V   E   A   D   D   V   A   T   Y   Y   C   Q   Q   S   N
721  GTG GAG GCT GAT GAT GTT GCA ACC TAT TAC TGT CAG CAA AGT AAT
     CAC CTC CGA CTA CTA CAA CGT TGG ATA ATG ACA GTC GTT TCA TTA

  +1  E   D   P   L   T   F   G   T   G   T   R   L   E   I   K
766  GAG GAT CCG CTC ACG TTC GGT ACT GGG ACC AGA CTG GAA ATA AAA
     CTC CTA GGC GAG TGC AAG CCA TGA CCC TGG TCT GAC CTT TAT TTT

                Spacer          Myc-tag
  +1  R | A   A   A | E   Q   K   L   I   S   E   E   D   L   N
811  CGG |GCG GCC GCA |GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT
     GCC |CGC CGG CGT |CTT GTT TTT GAG TAG AGT CTT CTC CTA GAC TTA

                Spacer    His tag
  +1  G | A   A | H   H   H   H   H   H   *   *
856  GGG |GCG GCA|CAT CAC CAT CAC CAT CAC TAA TAA
     CCC |CGC CGT|GTA GTG GTA GTG GTA GTG ATT ATT
```

Fig. 34-2

PelB-leader

```
+1   M   K   Y   L   L   P   T   A   A   A   G   L   L   L   L
1  ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC
   TAC TTT ATG GAT AAC GGA TGC CGT CGG CGA CCT AAC AAT AAT GAG

                                          |VH
+1   A   A   Q   P   A   M   A  |E   V   Q   L   Q   Q   S   G
46 GCG GCC CAG CCG GCC ATG GCC |GAG GTT CAG CTT CAG CAG TCT GGA
   CGC CGG GTC GGC CGG TAC CGG |CTC CAA GTC GAA GTC GTC AGA CCT

+1   P   E   L   V   K   P   G   A   S   V   K   I   S   C   K
91 CCT GAG CTG GTG AAG CCC GGG GCC TCA GTG AAG ATT TCC TGC AAA
   GGA CTC GAC CAC TTC GGG CCC CGG AGT CAC TTC TAA AGG ACG TTT

+1   A   S   G   Y   A   F   S   S   S   W   M   N   W   V   K
136 GCT TCT GGC TAC GCA TTC AGT AGC TCT TGG ATG AAC TGG GTG AAG
    CGA AGA CCG ATG CGT AAG TCA TCG AGA ACC TAC TTG ACC CAC TTC

+1   Q   R   P   G   Q   G   L   E   W   I   G   R   I   Y   P
181 CAG AGG CCT GGA CAG GGT CTT GAG TGG ATT GGA CGG ATT TAT CCT
    GTC TCC GGA CCT GTC CCA GAA CTC ACC TAA CCT GCC TAA ATA GGA

+1   G   N   G   D   T   N   Y   N   G   K   F   K   G   K   A
226 GGA AAT GGA GAT ACT AAC TAC AAT GGG AAG TTC AAG GGC AAG GCC
    CCT TTA CCT CTA TGA TTG ATG TTA CCC TTC AAG TTC CCG TTC CGG

+1   T   L   T   A   D   K   S   S   S   T   A   Y   M   Q   L
271 ACA CTG ACT GCA GAC AAA TCC TCC AGC ACA GCC TAC ATG CAG CTC
    TGT GAC TGA CGT CTG TTT AGG AGG TCG TGT CGG ATG TAC GTC GAG

+1   S   S   L   T   S   V   D   S   A   V   Y   F   C   A   D
316 AGC AGC CTG ACC TCT GTG GAC TCT GCG GTC TAT TTC TGT GCA GAT
    TCG TCG GAC TGG AGA CAC CTG AGA CGC CAG ATA AAG ACA CGT CTA

+1   G   N   V   Y   Y   Y   A   M   D   Y   W   G   Q   G   T
361 GGT AAC GTA TAT TAC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC
    CCA TTG CAT ATA ATG ATA CGA TAC CTG ATG ACC CCA GTT CCT TGG

                                          |Leader
+1   S   V   T   V   S   S  |G   G   G   G   S   G   G   R   A
406 TCA GTC ACC GTC TCC TCA |GGT GGA GGC GGT TCA GGT GGG CGC GCC
    AGT CAG TGG CAG AGG AGT |CCA CCT CCG CCA AGT CCA CCC GCG CGG

                                          |VL
+1   S   G   G   G   G   S  |Q   I   V   L   T   Q   S   P   A
451 TCT GGC GGT GGC GGA TCG |CAA ATT GTT CTC ACC CAG TCT CCT GCT
    AGA CCG CCA CCG CCT AGC |GTT TAA CAA GAG TGG GTC AGA GGA CGA
```

Fig. 35-1

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  +1  S   L   A   V   S   L   G   Q   R   A   T   I   S   C   R
496  TCC TTA GCT GTA TCT CTG GGG CAG AGG GCC ACC ATC TCA TGC AGG
     AGG AAT CGA CAT AGA GAC CCC GTC TCC CGG TGG TAG AGT ACG TCC

  +1  A   S   K   S   V   S   T   S   G   Y   S   Y   M   H   W
541  GCC AGC AAA AGT GTC AGT ACA TCT GGC TAT AGT TAT ATG CAC TGG
     CGG TCG TTT TCA CAG TCA TGT AGA CCG ATA TCA ATA TAC GTG ACC

  +1  Y   Q   Q   K   P   G   Q   P   P   K   L   L   I   Y   L
586  TAC CAA CAG AAA CCA GGA CAG CCA CCC AAA CTC CTC ATC TAT CTT
     ATG GTT GTC TTT GGT CCT GTC GGT GGG TTT GAG GAG TAG ATA GAA

  +1  A   S   N   L   E   S   G   V   P   A   R   F   S   G   S
631  GCA TCC AAC CTA GAA TCT GGG GTC CCT GCC AGG TTC AGT GGC AGT
     CGT AGG TTG GAT CTT AGA CCC CAG GGA CGG TCC AAG TCA CCG TCA

  +1  G   S   G   T   D   F   T   L   N   I   H   P   V   E   E
676  GGG TCT GGG ACA GAC TTC ACC CTC AAC ATC CAT CCT GTG GAG GAG
     CCC AGA CCC TGT CTG AAG TGG GAG TTG TAG GTA GGA CAC CTC CTC

  +1  E   D   A   A   T   Y   Y   C   Q   H   S   R   E   L   P
721  GAG GAT GCT GCA ACC TAT TAC TGT CAG CAC AGT AGG GAG CTT CCT
     CTC CTA CGA CGT TGG ATA ATG ACA GTC GTG TCA TCC CTC GAA GGA

                                                        | Spacer
  +1  R   T   F   G   G   G   T   K   L   E   I   K   R | A   A
766  CGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA CGG | GCG GCC
     GCC TGC AAG CCA CCT CCG TGG TTC GAC CTT TAG TTT GCC | CGC CGG

                    Myc-tag                             | Spacer
  +1  A | E   Q   K   L   I   S   E   E   D   L   N   G | A   A
811  GCA | GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT GGG | GCG GCA
     CGT | CTT GTT TTT GAG TAG AGT CTT CTC CTA GAC TTA CCC | CGC CGT

        His tag
  +1  H   H   H   H   H   H   *
856  CAT CAC CAT CAC CAT CAC TAA
     GTA GTG GTA GTG GTA GTG ATT
```

Fig. 35-2

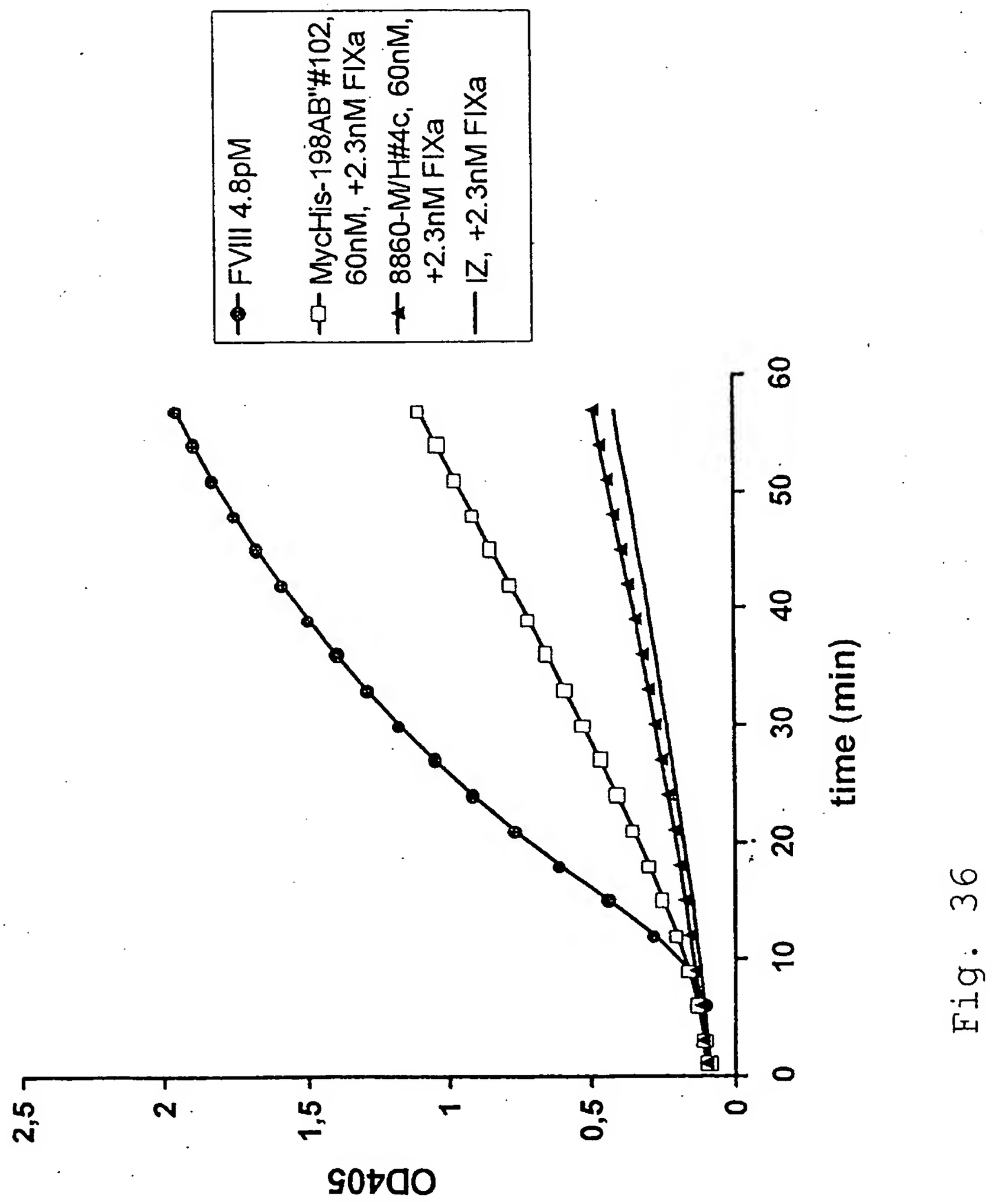
FVIII 4.8pM
MycHis-198AB"#102, 60nM, +2.3nM FIXa
8860-M/H#4c, 60nM, +2.3nM FIXa
IZ, +2.3nM FIXa
OD405
2,5
2
1,5
1
0,5
0
time (min)
0
10
20
30
40
50
60

Fig. 36